

## Certified Biological Nutritional Dental Professional Examination 2020

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1. Although the American Dental Association does not recognize “Biological Dentistry” as a specialty, individuals states have begun to license biological dentists.

- a. True
- b. False

### Licensure Overview

Licensure is a process every dentist must go through at least once during his or her professional life in order to practice dentistry. In the United States, licensure requirements vary from state to state and all applicants must meet three basic requirements: education, written examination, and clinical examination. The state dental board is the appropriate agency to contact for specific information about licensure requirements, the state dental practice act, or other licensure-related information. The Dental Licensure Map is helpful in finding initial licensure information by state. The information in this section is a brief summary of important facts to help dentists and dental students become more familiar with terms used and more informed about the licensure process.

### The Task Force on Assessment of Readiness for Practice

Ensuring patient safety and that each dentist meets professional standards for practice are the critical underpinnings of the dental licensure process. In support of this process a joint task force of the American Dental Association, the American Dental Education Association and the American Student Dental Association, released its groundbreaking report supporting the modernization of the dental licensure process, which is the culmination of over two years of research, discussion and collaboration between the organizations.

The report addresses two priority concerns with the existing licensure process in place in most states: 1) the use of single encounter, procedure-based examinations on patients as part of the licensure examination, and 2) mobility challenges that are unduly burdensome and unnecessary for ensuring patient safety. Download and read the full report.

### State Specific Licensure Information

As a member service, the ADA collects and summarizes state dental licensure information. All licensing jurisdictions are included. A quick reference to state licensure requirements and laws for dentists is available within the dental licensure by state map.

In the United States, the final authority on licensure requirements is the individual state. Though requirements vary from state to state, all applicants for dental licensure must meet three basic requirements; an education requirement, a written examination requirement and a clinical examination requirement.

Review the State Dental Licensure Requirements for U.S. Dentists section for more information.

Report of the Task Force on Assessment of Readiness for Practice

Licensure Overview <https://www.ada.org/en/education-careers/licensure>

## 2. Select the statement that is true:

- a. Epigenetics is the science of studying the genetic makeup of individual societies throughout the world.
- b. Genetic deformities are the cause of most chronic diseases in the US and are responsible for 60% of the prevalence of chronic disease.
- c. Our environment and the foods we eat are responsible for 80% of the prevalence of chronic disease in the US.
- d. The Human Genome Project has mapped out the entire human genome, which has been shown to be modifiable by surgical procedures.

### Role of Epigenetics in Biology and Human Diseases

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#### ABSTRACT

For a long time, scientists have tried to describe disorders just by genetic or environmental factors. However, the role of epigenetics in human diseases has been considered from a half of century ago. In the last decade, this subject has attracted many interests, especially in complicated disorders such as behavior plasticity, memory, cancer, autoimmune disease, and addiction as well as neurodegenerative and psychological disorders. This review first explains the history and classification of epigenetic modifications, and then the role of epigenetic in biology and connection between the epigenetics and environment are explained. Furthermore, the role of epigenetics in human diseases is considered by focusing on some diseases with some complicated features, and at the end, we have given the future perspective of this field. The present review article provides concepts with some examples to reveal a broad view of different aspects of epigenetics in biology and human diseases. DOI: 10.22045/ibj.2016.01

Keywords: DNA modification, DNA methylation, Gene expression

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#### INTRODUCTION

lassical definition by Conrad Waddington in the 1950s states “an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence”[1]. Based on our understanding of epigenetics, actual epigenetic definitions express that the whole DNA content is exactly the same in somatic cells of one species, while gene expressions patterns have distinct differences in various cell types that can be clonally inherited[2]. Epigenetic mechanisms can influence the gene activity at the transcriptional and post-transcriptional levels and/or at the translation level and post-translational modifications. Such epigenetic mechanisms with a potentially vast spectrum of consequences could result in more varieties of cell differentiations, morphogenesis, variability, and adaptability of an organism, which can be affected by both genetic and environmental factors[3]. Therefore, the field of epigenetics covers the modifications of DNA, DNA-binding proteins, and histones, which are important in making changes in chromatin structure without any change in the nucleotide sequence of a given DNA. Also, some of these alterations could be transferred between generations[4].

Epigenetic field and history Following Fleming’s discovery of chromosome in 1879, Thomas Hunt Morgan demonstrated that there is a genetic linkage between several *Drosophila* genes and X chromosome. Other studies have assigned individual genes to specific sites on the *Drosophila* chromosomes. In 1930, H. J. Muller carried out further genetic analyses and introduced a class of *Drosophila* mutations, which were connected to chromosomal rearrangements. He concluded that “chromosome regions affecting various characters at once, are somehow concerned, rather than individual genes or suppositious ‘gene elements.”[2,5,6]. In the past few decades, many investigations have shown that the epigenetic mechanisms are involved in

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regulation of all biological process in the body from conception to death. These functional mechanisms are involved in genome reorganization, early embryogenesis and gametogenesis, as well as cell differentiation. The interplay of DNA methylation and histone post-translational alterations, which cause as the result of regulatory proteins and non-coding RNAs, are key epigenetic players to rearrange chromatin into areas such as euchromatin, heterochromatin, and nuclear compartmentalization. Epigenetic signs may have long-term impressions, for instance, in learning and organizing memory or predispositions to different cancers. Incorrect epigenetic marks can result in birth defects, childhood diseases, or symptoms of diseases in other interims of life. Epigenetic mechanisms also regulate development and adaptations during the life of an organism, and their alterations may result in various disorders such as cancer. On the other hand, some epigenetic marks can be reversible, and this fact has encouraged many researchers to focus on epigenetic therapy[7]. In recent years, it has been demonstrated that DNA methylation, in some cases, can be irreversible[7-9]. This trait could be useful in complex features and challenging diseases such as memory function, psychological behaviors and injuries,

addiction, cancer, and other diseases that could not be explained just by genetic factors or the environment. Epigenetic modifications In a multicellular organism, the epigenetic changes enable different adult cells to express specific genes that are required for the existence of each cell type and transfer of information to the daughter cells. Epigenetic modifications often happen during an organism's lifetime; however, these changes can be transferred to the next generation if they occur in germ cells[10]. Paramutation, bookmarking, imprinting, gene silencing, X chromosome inactivation, position effect, changeable disorder or phenotypic severity, reprogramming, maternal attributes, carcinogenic processes, teratogenic effects, regulation of histone modifications, heterochromatin states and cloning are known to involve epigenetic processes. Three major epigenetic modification mechanisms are shown in Figure 1.

**DNA methylation and demethylation** DNA methylation status has high stability and serves as a special epigenetic memory of specific cells throughout all periods in the cell cycle. It may also regulate the expression and the activity of histone codes. Acceleration of DNA methylation at CpG sites is mediated by DNA methyltransferase enzymes such as DNMT1, DNMT3a, and DNMT3b. Inside the cells, S-adenosyl methionine act, as an important methyl group donor. In this sense folic acid and B12 play the determinant roles in re-methylation or the attraction of de-methylated form of S-adenosyl methionine through passive and active mechanisms[11,12]. A number of studies have demonstrated that these effective factors could change DNA methylation patterns and alter the levels of gene expression[13,14]. Studies have also confirmed that the nutritional status in the early years of life could affect DNA methylation pattern and gene expression levels in adulthood[15]. Furthermore, the methylation patterns in CpG sequences at cytosine residues can be heritable and act as tissue- and species-specific features. It is interesting that 70% to 80% of human DNA in CpG sequences are usually methylated[9,16], and correlations between methylation and expression levels in cis and trans have been reported[17]. Totally, DNA methylation, as a very impressive epigenetic agent, could influence the development of mutations, DNA faultless and durability, gene expressions, and chromatin modifications.

**Histone and non-histone modifications** Histone modification is one way of gene regulation through chromatin remodeling and includes acetylation, methylation, phosphorylation, ribosylation, ubiquitylation, sumoylation, and citrullination. Acetylation has been studied frequently in different investigations, and it has been shown to be mediated by five families of mammalian histone acetyltransferase enzymes[18]. Another group of molecules, known to act as non-histone modifiers, is RNA transcripts, which are necessary to maintain the activity of genes (directly or indirectly). For example, hepatocyte nuclear factor 4 increases the special gene transcription level, and MyoD has similar effects on muscle-specific genes[19]. miRNAs are about 17 to 25 nucleotides and are considered as a member of the noncoding RNAs that can mediate a large number of biological activities[20]. It has been demonstrated that the expression of miRNAs in a cell can regulate functions of about 200 messenger RNAs as their targets[21]. miRNAs can also regulate about 60% of protein-coding genes in human[22], and many miRNAs are epigenetically adjusted by methylation in CpG islands or histone modifications or both of them[20,23]. Structural inheritance is another non-histone mechanism of inheritance. Experimentally, it has been shown that

altered cellular patterns are inherited to the next generation of cells, and it appears that the present structures act as templates for new structures[24].

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Fig. 1. Three major epigenetic modification mechanisms. A) DNA methylation. DNA methylation is mediated by DNA methyltransferase enzymes at CpG sites. It can also decrease gene expression by reducing the binding of transcription factors or increasing the binding of methyl-CpG binding proteins[11,12,59,100]. B) Histone modification. Histone acetylation, particularly in lysine residues of histone tails, is an important histone

modification that can accelerate binding transcription factors and then gene expression beside DNA demethylation[18,59]. C) miRNA. The formation of miRNA begins in nucleus and continues in cytosol that can perform a mechanism to regulate gene expression in mRNA level[101]. mRNA level[101].

Inactive gene  $\rightarrow$  Gene expression

Inactive gene  $\rightarrow$  Gene expression

Binding of methyl-CpG binding proteins, Which recruit HDACs and co-repressors

5'-Methylcytosine

Cell chromatin

S-adenosylhomocysteine (SAH)

Block by follate, vitamin B6 and vitamin B12 deficiency

S-acenosylmethionine (SAM)

DNMTs

HATS

Un-methylated cytosine

Histone Tail

Acetyl

Cell chromatin

No methylation/Histon acetylation

Transcription factors complex

Active gene  $\rightarrow$  Gene expression

Transcription factors can bind

Cytosol

Drosha (RNase)

Pre-miRNA

Nucleus  
 Dicer(RNase)  
 mi-RNAs duplex  
 Target mRNA  
 Guide RNA Reducing gene expression

mRNA mRNA

(B)

(A)

(C)

Heterochromatin Reduced binding of transcription factors  
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Also, evidence has confirmed the importance of positioning in addition to epigenetic modifications such as DNA methylation and hydroxymethylation, which play an important role in structural inheritance[25].

Mitotic gene bookmarking An important issue in interpreting epigenetic and genetic modifications is the concept of mitosis gene bookmarking. Mitosis can play an important role in remodeling the transcriptional landscape. This view confirms that bookmarking mechanisms provide flexibility to permit alteration in cellular fate or differentiation. The distinct kinetics of dissociation and re-association of factors during mitosis as well as varying stabilities of histone marks argue that multiple mechanisms control mitotic partitioning[26]. Mitotic chromatin is transcriptionally inactive[27] and is excluded from most of the factors such as transcription agents and RNA polymerases[26,28-32]. To correct and complete cell division, regulatory proteins should re-use their exact genomic targets to return gene transcription states appropriately[26,33-36]. Studies have shown that the exact post-mitotic restoration of suitable transcriptional patterns is affected by epigenetic marking of mitotic chromatin to prevent tragic regulatory results[37,38]. For example, certain histone and DNA modifications remain in mitotic chromatin [39-42]. DNA methylation keeps transcription silent until the completion of mitosis[23,43], whereas specific histone modifications do not show such a clear effect on gene expression, and there are more elusive states and relations. After mitosis, to reactivate different sites of genes, transcription factors have to find their appropriate sites, which are located in transcriptionally silent chromatin through mitosis process[26]. For instance, poly ADP-ribose polymerase-1 creates stable epigenetic marks in metaphase chromatin at the transcription start sites of many genes that are necessary to restart transcription after mitosis[28].

**Role of epigenetics in biological processes** Epigenetic modifications are a dynamic process reflecting a complex interplay between an organism and its environment. For example, the acetylation of lysine residues in histone subunits usually can result in promoting gene transcription, or the methylation of Lys9 or Lys27 of histone H3 is correlated with gene repression. However, the methylation of Lys4, Lys36, or Lys79 of H3 is ordinarily associated with gene activity[44]. The heritable quantity of gene expressions is about 30%, while this percentage is decreased to about 23% in cells grown in culture. Such associations between methylation levels and genetic variations have been demonstrated in several organisms, tissues, and populations[17,45,46]. Animal studies in rats[7] have shown that hippocampal-dependent contextual learning can elicit a remarkably persistent fear-related memory, and this behavior is dependent on denovo DNA methylation. The inhibitors of DNMTs could abolish fear-related memory by preventing DNA methylation. Other studies have suggested that histone acetylation and DNA methylation influence memory formation, and DNA methylation has a key role in the storage of longterm memories in cortical brain regions[7,17]. The role of methylation in biological clock has been an interesting discovery in recent years. Studies of 353 epigenetic markers (DNA methylation of CpG dinucleotide) in DNA have made it possible to predict the ageing of tissues[47]. Also, the role of methylation and acetylation during differentiation has been demonstrated by studying Oct4 promoter regions[48].

**Epigenetics and environment** Epigenome generally comprises all epigenetic modifications such as DNA methylation and histone modifications, as well as non-coding RNAs at any given point in time. The cell epigenome is dynamic and can be affected by genetic and environmental factors. Furthermore, epigenetic modifications can be reversible, which makes the genome flexible to respond to environment changes such as nutrition, stress, toxicity, exercise, and drugs[17]. In the winter of 1944/45 during World War II, food supply was reduced due to blocked food transport by Germans, and food delivery by boat was impossible because water canals were frozen. Famine and lack of sufficient vitamins and proteins in diets affected the whole population, especially pregnant women. Since then studies have been carried out on the long-term effects of this incidence on newborn babies and individuals under such conditions. The results of these studies showed that individuals born after the famine had a higher susceptibility to a subset of diseases, including schizophrenia, stress sensitivity, and obesity[17]. One of the nutritional components in food, which plays a major role in methylation, is folate. Folate can influence methionine production by homocysteine remethylation in the form of 5-methyltetrahydrofolate. It has been reported that folate defect or shortage can enhance colorectal carcinogenesis through hypomethylation of genomic DNA[12]. Stress is an important environmental factor. Recently, some studies have demonstrated that people with post-traumatic stress disorder, who were abused during childhood, exhibit different levels of DNA methylation and gene expressions in comparison to those who were not abused[49,50]. Also, maternal stress

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during gestation has been indicated to be associated with neurodevelopmental and psychiatric disorders. Long-term studies on children exposing to stress in utero have shown to be predisposed to psychiatric disorders because of an increase in the promoter activities of glucocorticoid receptor[17,51,52]. As a human, we are exposed to various environmental toxins on a daily basis, and this can affect our health through changes in our epigenome. *Listeria monocytogenes*, *Clostridium perfringens*, and *Streptococcus pneumoniae* have been shown to induce dramatic changes in acetylations of histones via the toxins they produce[53,54]. Arsenic exposure studies have been demonstrated to result in global DNA alterations and gene promoters methylation levels, histone acetylation, histone phosphorylation, and miRNA expressions. Such influences of arsenic exposure have been linked to epigenetic dysregulation and carcinogenesis[55]. One of the major effects of physical exercise is on epigenetic modifications that can be beneficial to health and cancer patients. Modifications in DNA methylation patterns as a result of physical exercise can increase the expression of genes involved in tumor suppression and decrease the expression levels of oncogenes. Studies have shown that DNA methylation patterns are different in cancer cells, and hypermethylations and hypomethylations have been observed in the promoter of tumor-suppressing genes and oncogenes. These modifications could result in uncontrollable growth leading to tumorigenesis[56,57]. In patients with type II diabetes, several genes have been reported to be hypermethylated in muscle, including peroxisome proliferator-activated receptor gamma and coactivator 1-alpha[58,59]. Some drugs, such as procainamide and hydralazine have been shown[60] to have an enhancing effect on antinuclear antibodies. In recent studies, it has been reported that women using oral contraceptive pills have a lower global DNA methylation levels when compared to those who do not use such pills[61].

**Epigenetics and human diseases** Methylation is a common and widely used mechanism for epigenetic modifications in cells. It has been shown to be correlated with many human diseases, including different cancers, autoimmune disorders, neurological disorders (Fragile X syndrome as well as Huntington, Alzheimer, and Parkinson diseases and schizophrenia). Also, it has been suggested that methylation can be considered for complicated diseases influenced by some secondary factors such as sex differences and age, which could change disorder severity[62].

**Cancer** Epigenetic modifications have a considerable effect on cancer. Hypermethylation of promoter regions in tumor suppressor genes can inactivate many tumor suppressor functions. Methylation levels also play an important role in cell divisions, DNA repair, differentiation, apoptosis, angiogenesis, metastasis, growth factor response, detoxification, and drug resistance[12]. Such features have promoted huge advances in the early detection of cancer using methylation levels. For example, hypermethylation of promoter regions in APC and RASSF1A genes are considered as common epigenetic markers for early detection of cancer[63]. Also, hypermethylation of TP53 promoter region has been reported as a common marker for evaluation of cancer development[64]. There are also some other types of epigenetic changes in cancer. In recent years, dysregulation of miRNAs has been confirmed in breast cancer, which has a potential to be used as diagnostic biomarkers[65]. Also, hyper- and hypo-methylation of several genes in breast cancer have been

confirmed[66]. Microsatellite instability, chromosomal instability, and CpG island methylator phenotype have been identified as three major mechanisms affecting gene function in colorectal cancer (CRC). Microsatellite instability occurs in 15% of CRCs, which can result in instability phenotype by mutated or methylated mismatch repair genes[67]. In a comprehensive analysis of CRC tumors in Iranian patients, Brim et al.[68] demonstrated a high microsatellite instability rate (18%). From 15 known methylation target genes, APC2, PTPRD, EVL, GPNMB, MMP2, and SYNE1 were found to be methylated in most samples, which can be potentially used as specific clinical and pathological markers of CRC in this population[68]. The pathogenesis of CRC has been reported to be controlled by miRNAs, which can act as regulators of oncogenic and tumor suppressor pathways, responsible for the development of cancer. It has been confirmed that different miRNAs can be useful as biomarkers and are potentially applicable in prognosis evaluation and the detection of CRC stages[65]. It has been also observed that in the absence of O6-methylguanine-DNMTs activity as a DNA repair protein, the specific genes, such as K-ras and p53, might be accumulated by G-to-A transition. Furthermore, hypermethylation near the methylguanine-DNMT start codon in the specific locus is critical for cancer progression, which may have a prognostic value in CRC patients[69]. It has been indicated that miRNAs play an important role in many types of cancer: acute myeloid leukemia, acute lymphocytic leukemia,

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chronic myeloid leukemia, chronic lymphocytic leukemia, endometrial carcinoma, gastrointestinal cancer, lung cancer, bladder cancer, thyroid tumors, and esophageal adenocarcinomas. Hence, the potential applications of miRNAs in diagnosis and prognosis of these cancers would be highlighted in the near future[65]. Isocitrate dehydrogenase 1 (IDH1) and IDH2 genes are frequently mutated in low-grade gliomas, denovo acute myeloid leukemias in adult and in the subsets of chondrosarcomas and lymphomas. Interestingly, high correlation between histone and DNA methylation phenotype in IDH mutant gliomas has been reported[18]. In Tables 1, 2, and 3, epigenetic modifications in different types of cancer are shown.

Autoimmune diseases Natural and normal functions of immune system depend on self-tolerance, and self-tolerance deficiency can result in autoimmunity. Autoimmune disease concordance studies in both monozygotic and dizygotic twins have suggested a role for epigenetic factors. Epigenetic homeostasis failure, as a response to environmental agents, can result in gene expression changes in specific differentiated cells leading to dysregulated self-tolerance[70]. The immune system and target organ are two main players in an autoimmune disease process and the epigenetic modifications of these players could have roles in disease development. Many functions of immune cells such as hematopoietic lineage, rearrangement of antigen-receptor, allelic exclusion, and inducible immune responses against pathogens are epigenetically controlled. The alterations of epigenetic mechanisms regulating immunological development could promote autoimmunity

disease[71]. Interestingly, the frequency of autoimmune disease occurrence is notably more in women, and the reason may be due to female sex hormones. The involvement

of second X chromosome in immune response and genetic predisposition to autoimmunity is suspected. Considering the lack of enough knowledge on the exact cause of these immune diseases, a role for epigenetic regulatory mechanisms is highly possible[70]. Furthermore, there are many examples for correlation between epigenetic modifications and autoimmune diseases. For example, in patients with rheumatoid arthritis, DNA hypomethylation of HDAC1 (histone deacetylase 1) and HDAC2 levels, hyperacetylation of histones H3 and H4, and hypomethylation of histone H3 at lysine 9 have been observed in synovial tissues. In addition, in patients with multiple sclerosis, the hypomethylation of DNA have been detected in central nervous system white matter in comparison to healthy individuals. In systemic lupus erythematosus, the main targets of autoantibodies are hypomethylated apoptotic DNA and modified histones[71]. Several studies have confirmed the role of epigenetics in allergic conditions, and asthma is considered as one of the most complicated diseases in this category. Evidence suggests that both asthma and epigenetic mechanisms are heritable, and 36–79% of heritable, familial asthma cases have non-Mendelian inheritance pattern in more than 100 genes[72-75], which covers only a small portion of the disease etiology[73]. Interestingly, asthma and epigenetic modifications have been shown to be transferred from affected mother more than affected father in parental origin features[76], which can be a result of immune interactions between the fetus and the mother[77]. Utero exposures can affect asthma as well as epigenetic modifications, and both features can be influenced by environmental factors[78,79]. Classically, allergens are considered in relation to factors such as smoking behavior[79-81], and studies have confirmed that these agents can change epigenetic marks in asthma[82].

Table 1. Promoter methylation in different types of cancer

Cancer type Gene Promoter methylation Reference  
Breast

RARB2, MSH2, ESR1B, AKR1B1, COL6A2, GPX7, HIST1H3C, HOXB4, RASGRF2, TM6SF1, ARHGEF7, TMEFF2, RASSF1, BRCA1, STRATIFIN, RASSF1A

Hypermethylation [102]

Gastric RUNX3 Hypermethylation [102] Liver CDKN2A Hypermethylation [102]

Esophageal APC Hypermethylation Colorectal SEPT9, hMLH1, CDKN2A/p16, HTLF,

ALX4, TMEFF2/HPP1, NGFR, SFRP2, NEUROG1, RUNX3, UBE2Q1 Hypermethylation

[103,104] Lung RARB2, RASSF1A, CHFR, STRATI-FIN, SHOX2, RASSF1A APC1

Hypermethylation [102]

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Table 2. Histone modifications in different types of cancer

Neurodegenerative and psychological disorders The parental allele-specific gene expressions along imprinted domains are brought about by specialized sequence elements called ‘imprinting control regions’ (ICRs). ICRs are located just on one of the parental copies whose function is regulating gene expressions through an allele-specific manner. Although DNA methylation is the best investigated epigenetic alteration at ICRs, methylation and acetylation of histones in ICRs have also been reported[83]. For putting methylation imprints onto the ICRs, DNMT3A is essential. After fertilization, through somatic maintenance, the allelic methylation changes are conserved during development. This process is complicated due to its link to the cell cycle and requires the proportional functions of multi-enzymatic complexes that could be affected by intrinsic and extrinsic agents[84]. Other important methylation modifications have been recognized in genes involved in the development of Alzheimer’s disease and schizophrenia. Distinct reduction in DNA methylation has been identified[85] in Alu and other repetitive elements in the genome that are exclusively related to the early phase of life. Also, a role for epigenetic modifications has been confirmed in psychiatric diseases such as Rubinstein-Taybi syndrome and addiction, Huntington’s disease, and Fragile X syndrome. It is now widely accepted that for normal function and neurodevelopmental features of the brain, the constancy of DNA methylation and histone modifications is essential, and their dysregulation may result in disease phenotypes[86]. The significant role of epigenetics in brain development and disease is due to the following factors: 1) plasticity of epigenetics during all periods of brain development and aging as well as dynamic regulation in neurons, 2) disordered chromatin organization in both early childhood and adult neurodegenerative disorders, and 3) rapid increase in chromatin modifying drugs demonstrated to have unexpected therapeutic potential for degenerative and functional disorders of nervous system. These factors have attracted a vast interest in chromatin-associated mechanisms of neurological diseases, and a new field of study called ‘neuroepigenetics’[87] have been established. Numerous reports have pointed the association of DNA methylation with neurodegenerative diseases. Regulation of H3K4 methylation proteins are considered as an influencing factor in neurodegenerative disease, and the inactivation of histone demethylase enzymes can result in different disorders such as autism, Rett syndrome, and X-linked mental retardation[88]. In Table 4, some mental and neurological disorders are listed alongside their epigenetic aberrations.

Addiction The worldwide estimation of taking opioids (opiumlike substances) is about 13.5 million people of which 9.2 million use heroin[89]. Family, twin, and adoption studies have presented a large amount of evidence that indicates genetic backgrounds have an important role in addiction disease. A Two- to four-fold difference in types of substance abuse disorders have been reported in monozygotic in comparison to dizygotic twins and also different genetic variations have been linked to the various types of substance dependency[90]. An interesting picture emerged from most of drug addiction genetic studies emphasizes the importance of environment factors besides genetic determinants. Epigenetic changes the responses to the needs of an organism in diverse environmental

conditions through adaptive alterations. Epigenetic events and processes are usually used to incorporate the maintenance of neuroplastic changes, which are correlated with Cancer type Type of histone modification Lung adenocarcinoma Up-regulation of  $\alpha$ -2 glycoprotein 1 in consequence of global histone acetylation[105] Non-small cell lung Global H3 deacetylation[106]

Gastric

Global H3K9 trimethylation[107] Silencing of RUNX3 in the consequence of increased H3K9 dimethylation and decreased H3 acetylation[108]

Prostate

Global H3K9, H3K18, and H4K12 acetylation and H4K3 and H3K4 dimethylation[109]

Activation of PTEN, CYLD, p53, and FOXO3a by modulating histone H3K9 methylation and deacetylation[110] Colorectal Global H3K9 deacetylation[111] Pancreatic Acetylation of histone H3 promoter region of C/EPB $\alpha$ [112]

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Table 3. miRNA changes in different types of cancer[3,65,113]

learning and memory[91]. Dysregulation of epigenetic machinery, as the results of substance exposures, can lead to drug-seeking behavior and relapse of substance dependence. For instance, neuronal activation can alter DNA methylation of the Brain-derived neurotrophic factor (BDNF) promoter at cAMP response elements (CRE) binding sites inducing gene expressions[92]. The crucial role of BDNF was bolded in neural and behavioral plasticity in chronic opiate exposure through a steady down-regulation of exon-specific Bdnf expression in the ventral tegmental area. Special epigenetic changes, such as histone modifications, have been reported to mediate Bdnf gene activities in chronic morphine exposure[93,94]. Other observations in rats during forced abstinence from morphine have shown a significant H3 acetylation increase in the BDNF promoter II and histone H3 methylation changes in ventral tegmental area[95]. Other studies have demonstrated that the prolonged drug exposure leads to widespread transcriptional changes of genes with diverse cellular functions. This type of change is different from the changes observed in the early stages of drug-induced neural adaptive processes accompanied with specific changes in early response genes and signal transduction pathways[96].

Altogether, studies have indicated that the etiology of initiation, continuation, and relapse of substance dependence will be better understood considering epigenetic factors, which regulate multiple interacting neural signaling pathways that create enormous diversity in the continually developing brain[97,98]. In this review, we presented and discussed the results of many studies demonstrating that epigenetic mechanisms regulate gene expressions in different models and at various levels. The importance of epigenetics in different human disorders have attracted many interests in the last decade, especially in complicated disorders such as behavior plasticity, memory, cancer, autoimmune disease, addiction as well as neurodegenerative and psychological disorders. It is becoming

moreclear why many therapeutic approaches have failed in the past. It is hoped that by understanding epigenetic mechanisms involved in neurological and psychological disorders, more effective therapies would soon become available. Because of great potential, academia and industry have shown great enthusiasm to develop new epigenetic therapies. Drug development based on epigenetics is difficult and expensive like other novel drug targets. However, reversible nature of epigenetic

Cancer type Types of miRNA[ (+)=up-regulation/(-)=down-regulation] Oesophageal squamous cell carcinomas miR-21(+) Lung miR-17-92 (+) miR-34c, miR-145, and miR-142-5p, let-7(-) Primary head and neck squamous cell carcinoma miR-1, miR-133a, miR-205, and let-7d(-) bsa-miR-21(+) Gastric miR-106a(+) miR-433 and miR-9(-) Prostate miR-135b and miR-194(+) miR-23b, miR-100, miR-145, miR-221, miR-222(-) Melanoma miR-182(+)

Hepatocellular miR-18a(-)

Colorectal miR-let 7g, miR-21, miR-20a, miR-17-19 family, miR31, miR 135, miR-181b, and miR 200c (+) miR-34, miR-let7, miR-143, miR-145, miR-133b, and miR-126(-)

Bladder

miR-2 23, miR-26b, miR-221, miR-103-1, miR-185, miR-23 b, miR- 203, miR 17-5p, miR-23, miR-205(+) miR-29c, miR-26a, miR-30c, miR-30e-5p, miR-45, miR-30a-3p, miR-133a, miR-133b, miR-195, miR-125b, and miR-199a (-) Breast miR-21, miR-155, miR-23, and miR-191(+) miR-205, miR-145, miR-10b, and miR-125b (-)

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Table 4. Summary of epigenetic aberrations reported in mental diseases  
 Disease Epigenetic change (tissues) Ref. Fragile X syndrome Hyper-methylation at the FMR-1 gene with an expanded (CCG)<sub>n</sub> repeat [11] Huntington Histone modification in HDACs and histone KDM5D/Kdm5d [88] Rett syndrome Mutation in the gene encoding MeCP2 [11] Autistic patients and their parents Abnormal trans-methylation, trans-sulfuration metabolism, genome-wide DNA hypo-methylation and elevated blood homocysteine level (blood) [11] Down syndrome miR-99a, let-7c, miR-125b-2, miR-155, and miR-802 up-regulation [113] SCZ DNA hyper-methylation of the RELN promoter and SOX10 promoter (brain) [11] SCZ and BD DNA hypo-methylation of the MB-COMT promoter (brain) [11]

SCZ

Histone 3 lysine 4 hypo-methylation at the GAD1 promoter due to mixed-lineage leukemia 1 gene dysfunction (brain)

[11] SCZ (male) DNA hyper-methylation of the WDR18 gene (brain) [11] SCZ (male) Global DNA hypo-methylation (blood) [11] SCZ & Psychotic BD DNMT1 hyperexpression and increase in SAM content (corticalinter-neurons) [11] Bipolar II DNA hypo-methylation of PPIEL gene (blood) [11] BD (female) Hypo-methylation of RPL39 (brain) [11] Dementia Hyper-methylation of circadian genes, PER1 and CRY1 (blood) [11] Alcoholism DNA hyper-methylation of alpha synuclein promoter, HERP gene promoter and dopamine transporter gene (blood) [11]

SCZ, schizophrenia; BD, bipolar disorders

modifications has made therapeutic applications a possible alternative approach in the near future. DNA methylation inhibitors act against various cancers and also psychiatric diseases such as schizophrenia and bipolar disorders. For example, azacytidine and decitabine, as DNMT inhibitors, modulate epigenetic effects while toxicity and limited chemical stability of these drugs restrict their use in cancer therapy. In addition, histone deacetylase (HDAC) inhibitors are used in cancer therapy, cardiac hypertrophy, and heart failure and have been indicated to possess neuroprotective effects on cellular and animal models of Parkinson's disease. In oncology, RNAi plays an important role as a target of epigenetic drugs. For instance, in human CRC, the upregulation of miRNA135b is common, while in cervical cancer, miR-21, miR-126, and miR-143 are commonly upregulated[99]. Recent new data and knowledge relating to the importance of epigenetics in different human disorders promise a vibrant future for epigenetics research. The new research will integrate high-throughput sequencing technologies and the sophisticated algorithms to analyze the large amount of data produced by sequenced epigenomes. The epigenomic data will provide a chance to discover new epigenetic marks and their functions in different types of tissues, early development, and disease states. The association of epigenetic marks with specific diseases can help the development of tools to diagnose patients and measure the severity of a disease. Although there are issues with specificity and efficacy of many drugs being tested in animal models, further research on the epigenetic mechanisms will surely help the development of better therapeutic pathways and agents in the near future.

### 3. Select the statement that is false:

- a. Dental offices today can use simple urine tests to determine compatibility of dental materials.
- b. Dental offices today are able to determine the biocompatibility of most dental materials through simple blood tests.
- c. Titanium implants can create a cytokine immune response that may create systemic chronic inflammation.
- d. Frequent and repeated use of in-home bleaching trays can cause systemic oxidative stress.

How do I obtain testing?

Depending on your local laws testing is obtained through a physician, dentist or other healthcare provider licensed to order blood work in your area. Some states allow for ordering services to assist you in obtaining testing, we use Betterlabtestsnow.com as our partner where allowed by law. A blood draw is required to prepare a specimen for testing. The specimen is then shipped overnight to Clifford Consulting & Research, Inc. with the included shipping materials provided in the kit.

back

Is the test Prescriptive (who can order?)

Yes, the Clifford Materials Reactivity Test is a blood based test that requires a prescription in many states and countries. Local laws determine who may order blood work in your area. In some states a ordering service may be used, in such cases we have partnered with Betterlabtestsnow.com. Our policy for offering service is to work through Physicians, Dentists, and licensed professionals or through Betterlabtestsnow.com where allowed by law.

back

Do I need to fast or alter my diet for testing?

No, our lab testing is not effected by diet or fasting.

back

Will my medications interfere?

Most medications, including antibiotics and antihistamines, will not effect our testing. If you have been on a long-term treatment plan that includes cortical-steroids or immunosuppressive medications, please consult with your healthcare professional before ordering the test.

back

How long will it take to get my reports back?

The results are returned to the ordering professional within 3 business days for paper copies (USA) and same day as testing for electronic copies of testing.

back

Can I have a copy of the results?

The results are automatically provided to the ordering healthcare professional. Please speak with your doctor to obtain a copy. You may also send us a records request form to obtain additional copies. There is an additional fee for these subsequent printed or electronic copies. [Click Here for the Form](#)

back

Do you bill insurance?

We do not bill insurance, accept insurance assignment, or respond to insurance inquiries regarding protected patient information. We do not participate with the US Medicare nor Medicaid programs. Although we cannot guarantee nor predict the coverage of your healthcare insurance carrier, we do provide a detailed receipt for testing services with procedural codes (CPT).

back

Do you test for Anesthetics?

Anesthetic susceptibility is not tested in the Clifford Materials Reactivity Test. This is an area of great interest in our current research. We do cover the major preservatives used in local anesthetics.

back

Where do I go to get the test prepared?

The blood sample is drawn by a healthcare professional or on their order through a local drawing facility. An additional fee may be charged for the blood draw and preparation by the drawing service which is not included in our testing fee.

back

Can I ship my specimen on Thursday or Friday?

It is best to ship your specimen Monday through Thursday to our laboratory for testing. We do ask that no specimens be shipped on Friday as there are no available personnel on the weekends to accept the shipment and store the specimen. After the serum is separated and transferred from the clotting tube to the transport tube, the serum specimens drawn after Thursday may be stored in a refrigerator or freezer until the following Monday.

back

Do I need to pay for specimen shipping?

The cost of shipping the specimen is covered by us when using the included shipping materials in the specimen shipping kit provided by our lab. Specimens sent using any other shipping document or shipping company must be paid by the sender. The return shipping cost of results to the ordering professional is covered by Clifford Consulting & Research, Inc. in the standard testing fee.

back

What does the test screen for?

The Clifford Materials Reactivity Test screens for existing systemic reactions associated with 94 chemical groups and compounds commonly found in dental and orthopedic restorative materials. The Clifford Materials Reactivity Test is not intended as an allergy detection test.

back

Has my specimen arrived at the lab?

Domestic specimen shipments may be tracked by the tracking number on the preprinted return shipping label by going to the internet and logging on to fedex.com. International specimen shipments may also be tracked through the shipping company used.

back

What Dentist or Physician offers this testing in my area?

Testing may be ordered by any dentist, physician, or healthcare professional licensed to order blood work in your area. We would be happy to work with your preferred

professional. Alternately, we may be able to help you find a professional from your area by using the referral databases listed on our patient resources page.

back

How long are the test results good for?

Results can and do change over time. For most relatively healthy adults results should be stable for at least 18 to 24 months. There may be some healthcare treatments or new material exposures that can change results for better or worse.

back

Your healthcare professional is the best source for information regarding the usability of the results over time.

How accurate is the test (are there false negatives or positives)?

As with any biological lab testing, both false positives and false negatives can occur. The accuracy and precision of the Clifford Materials Reactivity test is approximately comparable to testing used for evaluation of blood sugar and cholesterol. Based upon the results of multiple samples drawn over a period of several days, the accuracy is about 95% or greater. The precision of repeated testing from a single sample is about 97% or greater.

Abstract Osteolysis of bone following total hip replacements is a major clinical problem. Examination of the areas surrounding failed implants has indicated an increase in the bone-resorption-inducing cytokine, interleukin 1 $\beta$  (IL-1 $\beta$ ). NALP3, a NOD-like receptor protein located in the cytosol of macrophages, has been shown to signal the cleavage of pro-IL-1 $\beta$  into its mature, secreted form, IL-1 $\beta$ . Here we show that titanium particles stimulate the NALP3 inflammasome. We demonstrate that titanium induces IL-1 $\beta$  secretion from macrophages and this response is dependent on the expression of components of the NALP3 inflammasome, including NALP3, ASC, and Caspase-1. We also show that titanium particles trigger the recruitment of neutrophils and that this acute inflammatory response is dependent on the expression of the IL-1 receptor and IL-1 $\alpha/\beta$ . Moreover, administration of the IL-1 receptor antagonist (IL-1Ra) diminished neutrophil recruitment in response to titanium particles. Together, these results suggest that titanium particle-induced acute inflammation is due to activation of the NALP3 inflammasome, which leads to increased IL-1 $\beta$  secretion and IL-1-associated signaling, including neutrophil recruitment. Efficacy of IL-1Ra treatment introduces the potential for antagonist based-therapies for implant osteolysis.

Keywords Titanium; inflammasome; neutrophils; IL-1; NALP3

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## INTRODUCTION

With over one million total joint replacements performed every year (1), joint replacement surgery is a major advance in treatment of people with arthritis (2). It is estimated that six million total hip and knee replacements will be performed per year in the US by 2015

making this a significant health issue. Long-term studies of hip and knee replacements have indicated that loosening of joint replacements, as well as bone-loss surrounding the replacement, increases over time. Approximately 10–20% of patients who undergo joint replacement surgery will develop joint loosening requiring replacement of the joint (3, 4). Patients who develop significant inflammation surrounding a fixed implant, if left untreated, will eventually develop joint loosening and, in some cases, bone loss surrounding the joint. This type of aseptic inflammation is associated with activation of macrophages in the tissue surrounding the prosthesis and the destruction of bone. Studies have shown that over time, small wear-particles generated from implants become dislodged and are released into the surrounding area (4) where they can be phagocytosed by circulating monocytes and macrophages (1). This uptake of particles stimulates cells to release pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), prostaglandin E2 (PGE2), and interleukin-1 $\beta$  (IL-1 $\beta$ ) (5–9). These cytokines, especially IL-1 $\beta$ , have been implicated in mediating osteoclast activation and/or bone resorption (10–12).

NALP3 [NACHT-, LRR-, and pyrin domain (PYD)-containing protein 3] has been shown to be involved in inflammatory responses to particulates including silica, asbestos and monosodium urate (MSU) crystals (13–15). Activation of the NALP3 inflammasome involves a conformational change in NALP3 into its active form, which then associates with its adaptor protein ASC (16) through PYD interactions. This complex leads to the recruitment of pro-caspase-1 through caspase-recruitment domain (CARD) interactions (17–19). Pro-caspase-1 is cleaved into its active form, Caspase-1. Active Caspase-1 cleaves proIL-1 $\beta$  into its active, secreted form, IL-1 $\beta$ . Secreted IL-1 $\beta$  can bind to and activate the IL-1 receptor (IL-1R), leading to a more robust pro-inflammatory response. Polymorphisms in NALP3 are linked to a spectrum of auto-inflammatory diseases with unchecked IL-1 production (20).

The role of the NALP3 inflammasome in the response to titanium wear-particles has not previously been characterized. We hypothesized that titanium particles activate a proinflammatory response in patients through activation of the inflammasome complex, leading to loosening and failure of the joint replacement. To assess this, we first determined whether injection of titanium particles into mice led to neutrophil recruitment, a hallmark of acute inflammation, and whether this neutrophil influx was dependent on the expression of IL-1R and IL-1 $\alpha/\beta$ . To examine the potential for an antagonist-based therapy, we also tested whether treatment of mice with the IL-1 receptor antagonist (IL-1Ra) could decrease neutrophil recruitment. Finally, in order to determine a role for the NALP3 inflammasome in titanium-induced inflammation, we examined whether titanium particles could activate IL-1 $\beta$  secretion in mouse and human macrophages deficient in key components of the inflammasome signaling complex: NALP3, ASC, and Caspase-1.

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METHODS AND MATERIALS

#### Reagents

Lipopolysaccharide (LPS), Nigericin, and poly(dA:dT) sodium salt were from SigmaAldrich (St. Louis, MO). CA-074-Me, Phorbol 12-myristate 13-acetate (PMA), and GeneJuice transfection reagent were from EMD Chemicals (Gibbstown, NJ). Commercially pure

titanium particles (Alfa Aesar, Ward Hill, MA) were autoclaved in H<sub>2</sub>O, washed with 100% ethanol, dried under UV light in a laminar flow hood, and resuspended in sterile PBS for subsequent studies. Particles were determined to have <1EU/ml of endotoxin using the Pyrochrome LAL endotoxin assay (Associates of Cape Cod, East Falmouth, MA). Particles (n=6708) had a median diameter of 1.86  $\mu$ m with a mean diameter of 3.805  $\mu$ m (SD = 6.065  $\mu$ m) as determined by confocal microscopy and (NIH) ImageJ software.

#### Mice

C57BL/6 (WT) and IL-1R-deficient (IL-1R KO) mice were from Jackson Laboratories (Bar Harbor, ME). CD14 KO and MD2 KO mice were a gift from D. Golenbock (UMass Medical School). IL-1 $\alpha$ / $\beta$  KO mice were a gift from Y. Iwakura (University of Tokyo). All mouse strains, age and sex-matched with appropriate controls, were bred and maintained in the animal facilities at the University of Massachusetts Medical School. All experiments involving live animals were in accordance with guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institutional Animal Care and Use Committee.

Intraperitoneal (i.p.) injections Mice were injected i.p. with sterile PBS (500  $\mu$ l), 4% Thioglycollate (1 ml), or 30 mg titanium particles (600  $\mu$ l at 50 mg/ml). For IL-1Ra studies, mice were injected subcutaneously with 200  $\mu$ l (1 mg/kg final dose) of IL-1Ra (Anakinra; Amgen, Thousand Oaks, CA) in 2% Hyaluronic Acid carrier (Calbiochem, EMD, Gibbstown, NJ) 2 h prior to and 1h following titanium injections. Mice were sacrificed by isoflurane inhalation followed by cervical dislocation. Peritoneal exudate cells (PECs) were isolated 16–18 h after injections as previously described (21).

Flow cytometric analysis To enumerate neutrophils, PECs ( $1 \times 10^6$ ) were incubated with anti CD16/CD32 monoclonal antibody (clone 2.4G2; BD Biosciences, San Jose, CA) for 30 minutes to block Fc $\gamma$ RIIB/III receptors and stained with Ly6G-FITC (BD Biosciences) and 7/4-Alexa647 (AbD Serotec, Raleigh, NC) for 30 minutes at 4°C. Following staining, cells were washed with PBS and analyzed on a LSRII (BD Biosciences). Neutrophil numbers in PECs were calculated by multiplying total cell numbers by the percentage of Ly6G+, 7/4+ cells. Data were acquired by DIVA (BD Biosciences) and were analyzed with FlowJo 8.8.6 software (Tree Star Inc., Ashland, OR).

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#### Cell culture

Immortalized mouse macrophages from WT, NALP3-deficient, ASC-deficient, and Caspase 1-deficient mice were made as previously described (13) and grown in DMEM supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and 1% Penicillin/streptomycin at 37°C with 5% CO<sub>2</sub>. Cells were plated in granulocyte macrophage colony-stimulating factor (GM-CSF) (1 ng/ml; eBioscience, San Diego, CA)-containing media for 18 h prior to stimulations. THP-1 cells (ATCC, Manassas, VA) were differentiated into macrophages with 10 nM PMA for 48 h and grown in RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, 1% Penicillin/streptomycin, 50  $\mu$ M  $\beta$ -Mercaptoethanol, and 10 mM HEPES at 37°C with 10% CO<sub>2</sub>.

Cell stimulations For particle stimulations, mouse and human macrophages ( $4-5 \times 10^5$ ) were primed for 3 h with LPS (100 ng/ml) to up-regulate pro-IL-1 $\beta$  expression or left unprimed (Media), then stimulated with titanium particles (Ti) at given concentrations

(mg/ml), transfected with 400 ng of poly(dA:dT) (dA:dT) using GeneJuice, or treated with Nigericin (5 mM) for an additional 6 h (mouse) or 18 h (human).

RNA interference Differentiated THP-1 macrophages were transfected with siGENOME SMARTpools (40 nM) against human NALP3 or ASC or non-targeting (NT) siRNA #2 using DharmaFECT 4 transfection reagent for 72 h. Cells were then stimulated as described above. All siRNA reagents were obtained from Dharmacon (Thermo Fisher Scientific, Lafayette, CO).

#### ELISA

Cell culture supernatants were assayed for murine or human IL-1 $\beta$  or IL-6 with ELISA kits from BD Biosciences according to the manufacturer's instructions.

Scanning Electron Microscopy (SEM) Macrophages were plated at 50–60% confluency in plastic tissue culture dishes. The following day, titanium particles (100  $\mu$ g/ml) were added to cells for 30 minutes at 37°C. Cells were then fixed and prepared as described in (22).

Statistical Analysis An unpaired, two-tailed Student's t-test was used to determine statistical significance of independent experiments where two groups were compared (Figures 1a–e). When more than two groups were compared, a one-way ANOVA (Figure 1f, 2, 3, 4b, 4c) or two-way ANOVA (Figure 4a) followed by Bonferroni's correction for post test comparisons was used. Values of  $p < 0.05$  were considered significant. Statistics were performed using GraphPad (Prism v5.0a) software.

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#### RESULTS

Titanium particles induce an IL-1 dependent neutrophil influx in vivo Mice injected with titanium particles exhibited a significant increase in neutrophils compared to injections of PBS (Figure 1a). As expected, increased neutrophil numbers were seen in mice injected with the positive control, thioglycollate. To control for the possibility that LPS or endotoxin present on the surface of the titanium particles was responsible for the apparent immune response to injections, we used MD2-deficient (MD2 KO) and CD14deficient (CD14 KO) mice. Both MD2 and CD14 facilitate LPS signaling through TLR4, and deficient mice are unresponsive to LPS (23, 24). Titanium injections were able to induce neutrophil influx in MD2 KO and CD14 KO mice at levels similar to WT control mice (Figure 1b, c), indicating that LPS did not contribute to neutrophil recruitment by titanium.

IL-1R has been shown to be required for neutrophil recruitment following exposure to stimulants in mice (13, 21, 25). To determine whether expression of IL-1R was required for titanium-induced neutrophil influx, IL-1R-deficient (IL-1R KO) mice were injected with titanium and compared to WT mice. Titanium injections were unable to induce a neutrophil influx in IL-1R KO mice (Figure 1d). In order to examine the role of IL-1 production, IL-1 $\alpha/\beta$  KO mice were injected with titanium. Deficient mice exhibited significantly lower numbers of neutrophils (Figure 1e), confirming a role for IL-1 associated signaling in the immune response to titanium wear-particles in vivo. Additionally, WT mice treated with IL-1Ra, the soluble receptor antagonist of IL-1, showed a significant decrease in neutrophil recruitment after injection with titanium, compared to control-treated WT mice (Figure 1f). Taken together, these results indicate a critical role for IL-1 in titanium-induced neutrophil recruitment.

Titanium induces IL-1 $\beta$  production in mouse and human macrophages WT immortalized mouse macrophages responded to titanium particles in a dose-dependent manner with high levels of secreted IL-1 $\beta$  (Figure 2a, c). Supernatants from macrophages that received an LPS prime only (Prime) did not exhibit an increase in IL-1 $\beta$  production. As a positive control, macrophages were transfected with double-stranded DNA, poly-dA:dT (dA:dT). WT macrophages produced a significant amount of IL-1 $\beta$  production in response to dA:dT stimulation. As additional controls, macrophages incubated with titanium particles alone produced low to un-detectable levels of IL-6, when compared to LPS stimulation, and IL-1 $\beta$ , when compared to primed cells (Figure S1), verifying that endotoxin levels associated with titanium particles did not play a role in observed cytokine responses.

A similar cytokine response to titanium particles was seen in PMA-differentiated THP-1 human macrophages. Titanium particles induced significantly higher levels of IL-1 $\beta$  secretion when compared to un-stimulated controls (Figure 2b). As a control, human macrophages exhibited mature IL-1 $\beta$  secretion in response to Nigericin, a known IL-1 $\beta$  stimulator. Titanium-induced IL-1 $\beta$  production was also dependent on particle concentration (Figure 2d). Together, these results demonstrate that both human and mouse macrophages respond to titanium particle stimulation with mature IL-1 $\beta$  secretion. St Pierre et al. Page 5

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Titanium particles are internalized in macrophages To visualize phagocytosis of titanium particles, immortalized mouse macrophages were incubated with titanium particles then analyzed through SEM. Titanium particles are highly internalized in macrophages (Figure 3a), with the majority of all titanium particles located within cells by 30 minutes.

Macrophage-associated particles had a median diameter of 1.02  $\mu\text{m}$  with a mean diameter of 1.21  $\mu\text{m}$  (S.D. = 0.79  $\mu\text{m}$ ) (Figure 3b), similar to that seen in failed joints (26).

Activation and release of cathepsin B following uptake and lysosomal destabilization is required for optimal inflammasome activation in response to silica crystals, alum, and amyloid- $\beta$  (13, 27). In order to determine whether cathepsin is required for inflammasome activation and subsequent IL-1 $\beta$  production in response to titanium, WT immortalized mouse macrophages were treated with the cathepsin B inhibitor CA-074-Me. Supernatants from cells pre-treated with CA-074-Me had significantly lower levels of IL-1 $\beta$  following titanium stimulation compared to untreated cells (Figure 3c). Treated and untreated cells produced comparable levels of the inflammasome independent cytokine, IL-6, in response to LPS, confirming that inhibition was inflammasome specific (Figure 3d). Together, these results indicate that optimal titanium-induced IL-1 $\beta$  production requires cathepsin B release.

Immune response to titanium requires the NALP3 inflammasome Supernatants from immortalized macrophages generated from NALP3- (NALP3 KO), ASC(ASC KO), and Caspase-1- (CASP KO) deficient mice exhibited undetectable levels of secreted IL- $\beta$  in response to titanium while IL-1 $\beta$  secretion from WT macrophages was readily detected (Figure 4a). Stimulation with dA:dT induces mature IL-1 $\beta$  production in a NALP3-independent manner (28). As expected, NALP3 KO macrophages were able to respond to dA:dT similar to WT cells, while ASC KO and CASP KO macrophages were unable to respond to dA:dT. As a control, WT and deficient cells were able to produce similar levels of the IL-6 in response to LPS stimulation for 6 hours (Figure 4b).

A similar result was observed in THP-1 human macrophages. Cells transfected with siRNAs against NALP3 and ASC exhibited a significant reduction in the secretion of IL-1 $\beta$  in response to titanium particles (Figure 4c) compared to no-siRNA or non-targeting (NT) siRNA controls. Together, these results implicate a role for components of the NALP3 inflammasome complex in the IL-1 associated response to titanium particles in both mouse and human macrophages.

**DISCUSSION** Our results show that titanium wear-particles induce IL-1 $\beta$  cytokine production in both human and mouse macrophages leading to IL-1 associated signaling and subsequent neutrophil recruitment (Figure 5). Although titanium is rarely used in bearing surfaces currently, titanium remains an important alloy used in many hip and knee replacements and understanding the immune response to titanium particles is an important contribution to our understanding the mechanisms of implant loosening and periprosthetic osteolysis. Studies have shown that among failed arthroplasties, patients with cemented titanium-aluminum

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vanadium prostheses had the highest amounts of IL-1 $\beta$  in serum, compared to patients with implants comprised of other metals, such as cobalt and chromium (6). Additionally, it has been demonstrated that patients with failed cementless total hip arthroplasties have significantly higher levels of TNF, IL-6 and IL-1 $\beta$  and increased macrophage numbers when osteolysis is evident (29). Our data support these findings and suggest a potential mechanism for increases in IL-1 $\beta$  surrounding failed prostheses.

A role for IL-1 associated pro-inflammatory cytokine production following titanium rod implantation has been demonstrated in a murine intramedullary model (9). Our studies support these observations and suggest that neutrophil recruitment may also play a role in inflammation. Previous reports have also shown that implantation of  $4 \times 10^8$  titanium particles (30 mg) onto the calvarium of mice leads to significant pro-inflammatory cytokine production and bone resorption after only one week (30). Our results show that peritoneal injection of 30 mg of titanium particles induces acute inflammation, as seen by neutrophil recruitment in mice within 16 h through IL-1 associated signaling. These results are consistent with other groups' findings that neutrophils are recruited/activated following stimulation with particles including silica, asbestos, and monosodium urate crystals (13, 14, 21, 25, 31). Our results support the hypothesis that titanium wear-particles activate a proinflammatory response in humans through IL-1 associated signaling (Figure 5).

Additionally, we have shown that neutrophil recruitment following titanium injections is significantly reduced in mice treated with the recombinant IL-1 receptor antagonist, IL-1Ra. IL-1Ra, also known as Anakinra<sup>™</sup>, has proven to be highly effective at decreasing bone erosion and joint space narrowing in rheumatoid arthritis patients (32–34). Studies have shown that retroviral delivery of IL-1Ra was able to decrease inflammation and inflammatory cytokine production in a murine air pouch model of osteolysis (35). It has also been shown that IL-1Ra treatment diminishes the amount of bone loss in ovariectomized rats and mice (36, 37). Polymorphisms within the IL-1 gene cluster associated with increased IL-1Ra mRNA expression have also been associated with decreased susceptibility to osteolysis after total hip arthroplasty (38). Along with these results, the ability of IL-1Ra to decrease neutrophilia following titanium injections in mice

introduces the potential for antagonist-based therapies for titanium and other wear-particle induced inflammation.

We further demonstrate that titanium particles induce IL-1 $\beta$  secretion through activation of the NALP3 inflammasome complex (Figure 5). The role of the NALP3 inflammasome in these events is consistent with previous observations in responses to other implant materials (39). Interestingly, a growing number of systemic inflammatory diseases, characterized by fever, anemia, and elevated levels of acute-phase proteins, have been linked to abnormalities in NLR signaling pathways. Previous studies have shown that certain patients have increased inflammation secondary to particulate debris and are more likely than others to develop osteolysis and subsequent mechanical loosening of the implant ultimately requiring revision joint replacement. Defining the underlying causes of osteolysis will influence the appropriate treatment and management of particulate induced osteolysis and possibly identify those patients at high risk for this condition pre-operatively before the primary total joint replacement. Taken together, our results increase the understanding of how implant materials, and particulate material in general, interact with the host immune system and

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provide further insight into the development of treatments for titanium-particulate debris induced inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The authors declare that they have no conflict of interest.

#### Abstract

**Background:** At-home bleaching is a technique characterized by the use of carbamide peroxide or hydrogen peroxide as a tooth whitening agent. However, no data exists regarding systemic safety of this technique. The aim of this study was to investigate the effect of at-home bleaching on serum redox homostasis.

**Methods:** Twenty-nine healthy volunteers who requested for tooth whitening participated in this study.

Specified bleaching trays were fabricated for the maxilla and mandible arches. Each participant was given two syringes containing 9% hydrogen peroxide gel to use for 30 min/night for 14 consecutive nights. To evaluate the redox status, the serum concentrations of malondialdehyde (MDA), total antioxidant capacity (TAC), and prooxidant-antioxidant balance (PAB) were measured. Blood samples were obtained in the morning prior to initiation of study and the morning after on expiration of the bleaching period. The collected data were analyzed using the t-test with confidence interval of 95%.

#### Accepted Article

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**Results:** Twenty-three subjects completed the study. The MDA, PAB, and TAC were increased significantly after the bleaching period (p-value = 0.001, 0.001 and 0.002, respectively).

**Conclusion:** At-home bleaching revealed the potential to disturb oxidant-antioxidant balance and induce oxidative stress.

**Clinical relevance:** unfavorable and potential side effects of at-home bleaching should be considered.

**Key words:** Dental bleaching; Hydrogen peroxide; Oxidative stress; Redox.

#### Introduction

In recent decades, tooth whitening techniques have grown rapidly mainly due to the esthetic reasons resulting to improvement in the undesirable appearance of teeth. Two main bleaching techniques are in-office and at-home methods. At-home bleaching technique can be done either dentist-supervised or self administered using over the counter (OTC) devices (1).

The bleaching process is based on a chemical reaction between pigmented molecules within the organic matrix of tooth and the oxidizing agents. In the course of the bleaching procedure, long-chain pigmented molecules are oxidized and split into smaller lighter molecules as well as other molecules including carbon, water, and oxygen (2). Different oxidizing chemicals were frequently applied to the tooth bleaching procedures. The main effective bleaching agent is hydrogen peroxide ( $H_2O_2$ ) as other materials break down to release this one. For instance, whitening products containing 10% carbamide peroxide release 3.5% hydrogen (1, 2). In the dentist-supervised at-home bleaching technique, also regarded as a conservative method of tooth whitening, the oxidative agents are held in contact with the tooth surface over a certain period in each day. In this method, the duration of exposure to the oxidizing agent depends on the concentration and type of bleaching agent (3).

A number of concerns over the safety of bleaching procedure have been raised by several researchers (4-7). Among the side effects reported for the tooth bleaching procedure, includes slight reduction of tooth enamel, a temporary increase in tooth hypersensitivity, along with irritation of the mouth soft tissue especially gingival tissue (8-11).

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In addition to the transient and local side effects of the bleaching agents, the long term and irreversible systemic effects of these agents including genotoxicity, cytotoxicity, and carcinogenicity have been investigated (12). Several animal studies have focused on these side effects. Ito et al. (13-16) reported the presence of adenoma and duodenum carcinoma along with hyperplasia following ingestion of 0.1% and 0.4% (w/v) of the hydrogen peroxide solution for 8 weeks in rats. Dahl and Pallesen (17) presented acute mucosal ulcerations in the stomach of rats following ingestion of carbamide peroxide in a dose-dependent manner. Timblin et al. (18) applied the hydrogen peroxide on human tracheal epithelium cells and observed over-expression of proto-oncogen-c-jun protein. However, conflicting reports over the long term effects of bleaching agents was observed along with no published report regarding these effects on humans. It should be noted that there is less clinical control in at-home bleaching using a night-guard in comparison to

the in-office bleaching. Additionally, the clinical control is least with over the counter devices without any dentist-supervision. Entrance of the oxidant agents (which are highly reactive) to the body circulation regardless of its entrance mean, could damage various cells and organs(19). Although there exists an intricate balance between oxidants (from internal metabolisms or exogenous sources) and antioxidants within the body cells, excessive oxidants could perturb this balance and lead to the oxidative stress (20); the result of which is damage to various organic compounds including lipids, proteins, and DNA (19). Moreover, high concentrations of the oxidants in comparison to the antioxidants can contribute to the pathogenesis of various types of cancers, cardiovascular diseases, and neurodegenerative disorders (21-23). Till date, there is no published clinical study that addresses the systemic effects of bleaching agents, making this study the first to consider the potential systemic adverse impacts of these components. The current study aimed to investigate if the oxidizing agents used in the dentist-supervised at-home bleaching technique could lead to redox perturbation and subsequent oxidative stress after the bleaching period.

## Materials and Methods

### Study population

Twenty-nine (19 females and 10 males) healthy volunteers who requested for tooth whitening and referred to the Department of Operative Dentistry at Mashhad Dental School participated in the study.

### All participants

signed a detailed informed consent and facilitated approval of this study by the Ethical Board of Mashhad

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University of Medical Sciences.

Dry mouth, enzymatic disorders, respiratory or digestive tract disorders, allergy to vinyl, tooth decay, exposed

root surfaces, broken teeth, enamel erosions, grinding para-function, poor oral hygiene, along with smoking

were the exclusion criteria. In addition, volunteers with any systemic disorder (hypertension, diabetes mellitus,

etc) as well as patients who had been using antioxidant supplements (vitamin C and vitamin E supplements)

were also excluded from this study. Participants were instructed against the use of fast foods or vitamin C/E

supplements during the study period.

### Bleaching Procedure

An alginate impression (Bayer, Leverkusen, Germany) was obtained to produce a negative mold of both maxillary and mandibular arches. Casts were poured subsequently with dental Stone powder (Tara, Iran). The bleaching tray was fabricated with 0.035 inch vacuum formed sheets using a vacuum tray-forming machine (Ultradent Products Inc., South Jordan, United States). The trays were trimmed to 2 mm of the gingival margins. Two 3-ml syringes of 9% hydrogen peroxide gel (Perfecta® Bravo® Tooth Whitening Gel, Premier Dental Products, United States) with close to natural pH were handed to each participant. The patients were instructed to place enough bleaching agent into the tray to cover the facial surfaces of the target teeth (which were visible during smiling, laughing, and talking) and then put the loaded tray on their teeth. Obtaining maximum benefit of the product and patient compliance, participants were asked to wear the bleaching trays for 30 min per night for 2 weeks (according to the manufacturer's instruction) every night after daily routine flossing and brushing. After the loaded tray was seated, the patients were instructed to remove any trace of excess bleaching material on gingival tissue to minimize gingival irritation and swallowing of the bleaching agents. Subjects were cautioned to discontinue the use of bleaching agents in cases of tooth hypersensitivity or gingival problems and call the research team immediately.

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### Blood sample

Five milliliters of venous blood sample was collected from all participants twice; once in the morning of the day of commencement of the bleaching procedure, and thereafter in the morning of the day after the last night of bleaching. After separating serum from the whole blood, samples were kept at -80°C until the time of assaying.

### Thiobarbituric Acid Reactive Substances (TBARS) Assay

The serum concentration of malondialdehyde (MDA), both as a product of lipid peroxidation and as a

biomarker of oxidative stress, was measured using a commercial kit (Cayman Chemical, Michigan, United States, Item Number 10009055) based on an established method(24). The assay was based on the formation of a pink colored complex between MDA and thiobarbituric acid under high temperature (100°C) and acidic conditions. Absorbance of the MDA-TBA complex was read at 532 nm and the concentration of MDA in samples was determined using a standard curve provided using 0 to 5 µM MDA.

#### PAB Assay

A method described by Alamdari et al. (25) was utilized to estimate the prooxidant-antioxidant balance. In summary, for plotting the standard curve, varying proportions of 250 µM hydrogen peroxide (as a representative of pro-oxidants) with 3 mM uric acid in 10 mM NaOH (as a representative of antioxidants) were mixed, implying that the ultimate oxidant sample contained only hydrogen peroxide with a concentration of 250 µM, while the ultimate antioxidant sample had only uric acid with a concentration of 3 mM. Thereafter, in each well of a 96-well plate, 10 µl of each sample, standard or blank (distilled water) were mixed with 200 µl of the working solution which had been prepared according to the protocol (Alamdari et al., 2008). After 12 min incubation in a dark place at 37°C, reactions were terminated through the addition of 100 µl of 2 N HCl to each well; thereafter, the absorbance of samples was read at 450 nm with a reference wavelength of 620 or 570 nm.

The values of the unknown samples were calculated according to the standard curve.

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#### Total Antioxidant Capacity (TAC) Assay

To estimate the serum total antioxidant capacity, a method described by Miller et al. (26) was used that is the basis of a commercial kit (Cayman Chemical, Michigan, United States, Item Number 709001). This assay was based on serum antioxidants capacity for preventing the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical to ABTS radical cation by Metmyoglobin. The bleaching effect of antioxidants on blue-green color of ABTS radical cation was found to be proportional to the total antioxidant capacity that was measured as a change in absorbance at 660 nm. The experimental results obtained were compared with that

of Trolox, a tocopherol analogue, and were presented as millimolar Trolox equivalents. All other chemicals were purchased from Sigma-Aldrich Company (Sigma-Aldrich Inc., Taufkirchen, Germany).

### Statistical Analysis

The data obtained before and after bleaching periods were collected. The normality of data distribution was evaluated using one-sample Kolmogorov-Smirnov test. The difference between two measurements was performed using paired-sample t-test or Wilcoxon Signed Rank test. In addition, due to sample size limitations, the effective variables' size was measured using Eta Squared test. SPSS version 11.5 software (SPSS, Chicago, IL, USA) was used with the confidence interval of 95%.

### Results

Twenty-three patients (16 females and 7 males) completed the study while six of the registered participants did not continue the project to the end. Two participants had moved away and were unable to participate in serum sampling sessions; also, others did not apply the bleaching agents according to specified standard protocol. Subjects used bleaching gels for  $14 \pm 2.1$  nights. The age of the subjects was  $29.4 \pm 9.5$  years old (ranging from 18 to 53). None of the subjects who completed the study reported any signs of tooth hypersensitivity or gingival problems during the study.

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The MDA concentration (TBARS), PAB, and TAC were all normally distributed in our measurements.

According to paired-sample t-test, serum concentration of MDA after bleaching ( $4.77 \pm 1.87 \mu\text{M}$ ) was

significantly higher than that of pre-bleaching samples ( $3.14 \pm 1.00 \mu\text{M}$ ) with a p-value of 0.001 (Fig. 1). The

effect size of TBARS test was observed to be 0.427.

For PAB assay, since the standard curve was plotted based on increasing concentrations of hydrogen peroxide,

the higher value of PAB indicated a higher degree of redox perturbation in favor of oxidants. In this study,

while the PAB value for pre-bleaching samples was  $77.84 \pm 14.40$ , a significantly higher value of  $115.36 \pm$

$26.18$  was noticed after bleaching (p-value < 0.001) (Fig. 2). The effect size of PAB test was found to be 0.820.

As shown in Fig. 3, serum TAC which is proportional to both total concentration and capacity of all antioxidants presented in serum, revealed an increased amount after bleaching ( $1.22 \pm 0.07$  mM) compared to pre-bleaching value of  $1.17 \pm 0.06$  mM; this increase was statistically significant (p-value=0.002). The effect size of TAC test was found to be 0.371.

### Discussion

Findings of the current research indicated that at-home bleaching resulted to significant increase in serum level of malondialdehyde. Despite the total antioxidant capacity being increased significantly, the prooxidant antioxidant balance was significantly shifted in favor of pro-oxidants. Consequently, systemic oxidative stress was observed at the end of the tooth bleaching period. With the limited sample size of the current study, further analysis was performed along with determination of the resulting size of each assay. Regarding the scale proposed by Cohen, the Eta Square value which equals and exceeds 0.16 is assumed as large effect size (27). As a result, the consequent size of TBARS, TAC, and PAB were altogether largely considered; implying that large proportion of variance in these assays is explained by the at-home bleaching procedure.

Determination of the lipid peroxidation level reveals the amount of free-radicals since they can potentially initiate the peroxidation reaction of polyunsaturated fatty acids. The product is unstable lipid peroxide which decomposes to various compounds including malondialdehyde (MDA) (24). Lipid peroxidation refers to a mechanism of cell injury and is an indicator of oxidative stress; the products of which play an important role in

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 wound healing process by activating the arachidonic acid cascade in plants (28). These products also help in killing bacteria and spores in tissue injuries (29). However, excessive lipid peroxidation leads to membrane rupture and cytotoxicity (30). Most disease conditions and toxins have been found to increase lipid peroxidation. The peroxidation products are responsible for further cytotoxicity (29). Animal studies along with a case report of a 16-month ingested hydrogen peroxide have shown the acute cytotoxicity of the bleaching

agents (12). Nevertheless, systemic oxidative stress in the dentist-supervised at-home bleaching procedure has been observed as the MDA serum level was increased.

MDA concentration has been reported as the most practical and precise technique in detecting oxidative stress (31). This marker has been used in investigating the role of oxidative stress in various diseases including chronic obstructive pulmonary disease (COPD), coronary heart disease, and diabetes mellitus (32-34). Tug et al. (34) reported that the level of MDA measured by means of TBARS was 3.46  $\mu\text{M}$  in healthy subjects, 5.22  $\mu\text{M}$  in post-exacerbation period of COPD, and 7.15  $\mu\text{M}$  during exacerbation period. Furthermore, Khan and Baseer (33) reported that the mean MDA concentration in healthy subjects and patients with the coronary heart disease was 2.24  $\mu\text{M}$  and 3.46  $\mu\text{M}$ , respectively. They assumed this increase as an indication of oxidative stress. In the present study, authors found comparable increase in the MDA concentration (3.14  $\mu\text{M}$  before and 4.77  $\mu\text{M}$  after bleaching) which could be interpreted to mean as systemic oxidative stress due to exposure to the bleaching agents.

Along with increasing lipid peroxidation reactions during bleaching, the total antioxidant capacity (TAC) was also increased; this shows the body's reaction toward entrance of toxic agents in circulation. However, the magnitude of the TAC increase was not as large as the magnitude of the increase in serum MDA concentration; hence, redox perturbation observably occurred in favor of oxidants indicated by the PAB assay.

In addition to some biological reactions as endogenous sources of free radicals, external sources are xenobiotics, ionizing radiation, UV light, some drugs, cigarette, fast foods, etc (35-37). However, the human body contains effective protective mechanisms against harmful effects of free radicals. Superoxide dismutase, glutathione peroxidase, catalase, the thioredoxin system, albumin, ferritin, bilirubin, and uric acid are part of the natural antioxidant systems (38, 39). The nutrients such as  $\alpha$ -Tocopherol (vitamin E) and ascorbic acid (vitamin C) also play an important role in the antioxidant activity against oxidants (39). In this study, to eliminate interference of the oxidant as well as antioxidant compounds with effect of the bleaching agents, a number of

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exclusion factors such as the consumption of vitamin C/E supplements as antioxidants, cigarette smoking, drugs, and fast foods were defined as the oxidant sources.

The imbalance between oxidative species and the antioxidant components, in which there exists an exceedingly higher sum amount of endogenous and exogenous oxidants, leads to the oxidative stress (19). In the present study, oxidative stress was observed after completion of bleaching period (2 weeks period). Although no side effects on short term oxidative stress could be detected in this study, according to the observed redox perturbation, safety of long term bleaching in cases of long term bleaching (that is, tetracycline discolorations) should be taken into consideration. Cerebral infarction, cardiac ischemia due to gas embolism, gastrointestinal emphysema, and lung edema has been reported in cases with hydrogen peroxide ingestion (40-42). Moreover, gastrointestinal malignancy, pathological alterations in pre-malignant lesions, and morphological changes in oral tissues due to local contact of whitening agents has been reported in animal and human studies (12).

Nonetheless, there is a gap between the animal or in vitro studies and human studies as no clinical trial study is present to investigate the possible systemic effects of the bleaching agents.

Although International Agency on Research on Cancer attributes no carcinogenicity risk to the professional use of the bleaching agents (43), a recent review suggests that long term use of hydrogen peroxide with high concentration could promote oral mucosa injury, genotoxicity, and carcinogenicity (12).

The observed findings of this study supported the later viewpoint as entrance of the oxidizing agents to blood circulation during the dentist-supervised at-home bleaching pertains to two possible mechanisms: swallowing small amounts of the hydrogen peroxide during application with night-guard and subsequent absorption through gastrointestinal tract; or local absorption through gingiva. Each pathway aggravates concerns over contact of agents with oral mucosa or GI lining and the possibility of genotoxicity, and carcinogenicity in cases with long term bleaching period (44). Moreover, free radical species react and oxidize cellular components, and further causes damage to lipids, DNA, and proteins (19). These damages could also subsequently result in a range of diseases that has been previously mentioned (21-23).

None of the subjects who completed the study reported any signs of tooth hypersensitivity or gingival problems during the tooth whitening period. It may be related to natural pH and thixotropic nature of this at-home bleaching agent as described by the manufacturer to ensure it stays in contact with the teeth.

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However, tooth bleaching is considered an important way to improve people's social life and also at-home bleaching is the most conservative as well as the most effective method of bleaching. Hence, the authors are currently performing another clinical trial to investigate whether this oxidative stress resulting from the at-home bleaching could be diminished via shifting prooxidant-antioxidant balance in favor of the antioxidants through the use of the antioxidants (vitamin C supplements) during the procedure. One of the limitations of the current study was that changes in redox status were not evaluated after the bleaching period to determine the time needed to fully recover from the oxidative stress.

#### Conclusions

Based on the limitations and results of the present study, it could be concluded that oxidative stress is a consequence of the dentist-supervised at-home bleaching technique. Although this stress is probably transient, probable side effects of the short term oxidative stress should be taken into consideration. Additionally, in cases with long term bleaching procedures or in situations with no dentist supervision as in over-the-counter tooth whitening products, the side effects may be significant hence the concern should be properly considered.

#### Clinical Significances

The redox imbalance due to oxidative stress with application of the tooth whitening agents may induce some probable harmful effects. Technical issues as well as careful education of patients on how to use the bleaching agents and trays should be seriously considered to minimize the possibility of oxidizing agents' leakage.

4. Dental x-rays have been proven to pose no threat to humans from their low dose ionizing radiation.

- a. True
- b. False

## INTRODUCTION

Dental diagnostic X-rays are an essential part of dental practice. Although radiation doses have been reduced due to the development of digital techniques, dental diagnostic X-ray imaging remains one of the most common types of radiological procedures that are frequently performed in dental clinics for oral examinations [1,2]. Dental diagnostic X-rays for certain types of examinations, including bitewings, full-mouth series, and panoramic views, are in common use. Considering the lifetime frequency of exposure to dental diagnostic X-rays, even a slight increase in health risk would be of considerable public health importance [3]. Dental X-rays expose patients to relatively low radiation doses. However, the survivors of the Hiroshima atomic bombings provide evidence of increased cancer risk from low doses of radiation [4]. Repeated exposure could also increase cancer risk [5]. Exposure to dental X-rays is associated with potential risk of cancer, which was revealed in previous studies [6,7]. In the head and neck region, cancer risks caused by exposure to dental X-rays have been discussed. Although many epidemiological studies have reported on the association between exposure to dental X-rays and meningioma risk, it is still controversial, as some studies have shown a lack of association [8-10]. A meta-analysis study proposed that there is no clear evidence of a significant association between exposure to dental diagnostic X-rays and the risk of developing meningioma [11]. Several studies have found an association between dental X-ray exposure and increased risks of brain cancer [12,13], tumors of the parotid gland [14] and breast cancer [15] and thyroid cancer [16,17]. In particular, thyroid cancer is one of the most common cancers in the worldwide, and the side effects from dental radiation exposure are likely to contribute to its incidence due to the location of the thyroid gland. Repeated exposure to dental X-rays may result in various health problems including head and neck tumors and various systemic problems. Thus, we conducted a systematic review of papers that reported an association between dental X-ray exposure and overall health risks be

Health effects from exposure to dental diagnostic X-ray

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The purpose of this review is to summarize the results of studies on of the association between exposure to dental X-rays and health risk. To perform the systematic review, We searched the PUBMED, EMBASE, and MEDLINE databases for papers published before

December 15, 2016. A total of 2 158 studies, excluding duplicate studies, were found. Two reviewers independently evaluated the eligibility of each study. The final 21 studies were selected after application of exclusion criteria. In terms of health outcomes, there were 10 studies about brain tumors, 5 about thyroid cancer, 3 about tumors of head and neck areas, and 3 related to systemic health. In brain tumor studies, the association between dental X-ray exposure and meningioma was statistically significant in 5 of the 7 studies. In 4 of the 5 thyroid-related studies, there was a significant correlation with dental diagnostic X-rays. In studies on head and neck areas, tumors included laryngeal, parotid gland, and salivary gland cancers. There was also a statistically significant correlation between full-mouth X-rays and salivary gland cancer, but not parotid gland cancer. Health outcomes such as leukemia, low birth weight, cataracts, and thumb carcinomas were also reported. In a few studies examining health effects related to dental X-ray exposure, possibly increased risks of meningioma and thyroid cancer were suggested. More studies with a large population and prospective design are needed to elaborate these associations further.

Keywords: Dental radiography, Health, Radiation exposure

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cause no previous reports have summarized these associations.

#### MATERIALS AND METHODS

The patient, intervention, comparator, outcomes (PICO) method was followed as a viable tool for the systematic review process [18]. The PICO method for this systematic review was as follows. The “P” referred to all patients, the “I” referred to dental X-ray exposure, the “C” referred to dental X-ray non-exposure, and the “O” referred to brain cancer, meningioma, thyroid cancer, leukemia, and other cancers.

Search strategy and data sources We searched the PUBMED, EMBASE, and MEDLINE databases and performed a manual search for papers. The databases were searched for all related paper published before December 2016. The paper published in all languages were selected. The following search terms were employed. (i) PUBMED: (“radiography, dental” [MeSH Terms] OR (“radiography” [All Fields] AND “dental” [All Fields]) OR “dental radiography” [All Fields] OR “dental x ray” [All Fields]) AND exposure [All Fields] AND (“brain neoplasms” [MeSH Terms] OR (“brain” [All Fields] AND “neoplasms” [All Fields]) OR “brain neoplasms” [All Fields] OR (“brain” [All Fields] AND “cancer” [All Fields]) OR “brain cancer” [All Fields]); (ii) EMBASE: ‘dental’/exp OR dental AND (‘x ray’/exp OR ‘x ray’) AND (‘exposure’/exp OR exposure); (iii) Manual search was carried out using the reference lists of papers included in the systematic review, on review papers about overall health.

Study inclusion and exclusion Two reviewers (S.Y.H, E.S.C.) independently assessed the eligibility of each study through the databases based on the predetermined selection criteria. Any disagreements were resolved through discussion. The inclusion criteria were as follows: (i) human study; (ii) health problems including cancers related to dental radiation exposure; (iii) the full text of the study was available. The exclusion criteria were

as follows: (i) Radiation dose assessment study; (ii) Radiation safety management study; (iii) Review articles; (iv) Letter and recommendation.

Data extraction Two authors (M.A.H. and H.Y.K) independently collected the following information: first author's name, year of publication, study design, dental diagnostic X-ray type, health outcome, significance, risk estimates, and their confidence intervals (CIs). We tried to summarize the significance of test results according to the types of health outcomes and types of the dental x-ray.

However, test results in some studies were expressed for more detailed categories such as age groups or frequencies of exposure and occurred either consistent which means that all test were significant or nonsignificant, or inconsistent which means that both coexisted. To solve the problem, the integrated significance was marked as having partial significance (PS) when only some of the characteristics were statistically significant, as having significance (S) when all the characteristics were significant, and as not having significance (NS) when all were nonsignificant.

Quality assessment We assessed the methodological quality of each study using the Newcastle-Ottawa scale (NOS) [19] which uses a star rating system. A full score is 9 stars, and a score range 5 to 9 stars is considered to be a high methodological quality while a score range 0 to 4 is considered to be poor quality. Nineteen case-control studies and one cohort study were assessed on the quality excluding one case-report study.

## RESULTS

A total of 2 158 studies, except for duplicate studies, were initially collected. The abstracts and titles of the 2 158 papers were assessed. The full-text of the final 21 studies that were selected through classifications of exclusion criteria were read (Figure 1). We assessed information provided by the reporting of case– controls (n=19), cohort studies (n=1), and case studies (n=1). These studies were published between 1997 and 2015. These studies were conducted in the following countries: the United States (n=12), Sweden (n=4), Taiwan (n=1), Australia (n=1), Kuwait (n=1), Japan (n=1), and Syria (n=1).

Quality assessment The quality of all included studies was summarized in Table 1.

Figure 1. Flow chart of identification of eligible studies to final inclusion.

Potential articles identified (n=3 486) PubMed (n=1 541) EMBASE (n=1 025) Medline (n=918) Hand search (n=2)

Records excluded based on titles and abstracts (n=2 137) Dose (n=243) Safety (n=66) Reviews (n=10) Other (n=1 818)

Duplicate records & only abstract excluded (n=1 328)

Relevant records screened (n=2 158)

Full-text articles reviewed (n=21)

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The median NOS score of the eligible studies was 4.0, 5.0, and 3.5 for meningioma and tumors in head and neck areas, thyroid cancer, and systemic health outcomes, respectively.

Health-related outcomes Brain tumors Table 2 shows 10 research papers on dental diagnostic X-rays and brain tumors. All were case–control studies. Five categories

Table 1. Newcastle-Ottawa Scale Quality Assessment of included studies (N=20) Type Author(year) Study design Selection Comparability Exposure Total Brain tumor Preston-Martin (1980) Preston-Martin (1983) Preston-Martin (1989) Neuberger JS (1991) Ryan P (1992) Rodvall y (1998) Longstreth w (2004) Claus E (2012) Han YY (2012) Lin MC (2013)



US Case-control 1 433/1 350 Full mouth Bitewing Panorama  
 Meningioma NS S S  
 Positive association (OR=2.0, 95%CI=1.4-2.9) Positive association (OR=3.0, 95%CI=1.6-5.6)  
 at frequent exposure, yearly or more  
 Han YY (2012) [25]  
 US Case-control 343/343 Any Vestibular schwannoma  
 S Strong positive association (OR=4.26, 95%CI=1.49-12.18)w  
 Lin MC (2013) [26]  
 Taiwan Case-control 4 123/16 492 Any Any  
 Benign Brain tumor Malignant Brain tumor  
 S NS  
 Positive association (OR=1.39)

aPS: partial significance S: significance NS: no significance

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alized or specified the types of dental diagnostic X-rays, while the other five did not. Two of these studies also included the panorama and bitewing types of examinations. Detailed analyses according to age groups or exposure frequencies were performed in five studies examining the correlation between the full-mouth examination type and brain tumors. There were seven papers on meningiomas, three on gliomas, one on acoustic neuroma, and one on vestibular schwannoma, while two papers mentioned unspecific brain cancer. Seven papers reported a significant positive association and three presented a partially positive significant association, while eight had nonsignificant results. One paper reported a negative association between full-mouth X-ray exposure and glioma. The association with meningioma was significant in 5 of 7 studies, while it was nonsignificant in the remaining 2 studies. Inconsistent results were reported on the association with glioma. A strong positive association was found in relation with vestibular schwannoma, while not with acoustic neuroma. The full-mouth examination type increased the risk of brain tumors significantly in three of the four studies.

Thyroid cancer There were five research findings related to thyroid cancer (Table 3). As far as research design was concerned, four were case-control studies, while one was a cohort study. The five

studies did not specify dental diagnostic X-ray types. In four of the five studies, there were significant correlations between dental diagnostic X-rays and thyroid cancer. One of these studies categorized the subjects by occupation and confirmed the correlation between dental practice and thyroid cancer.

Tumors in head and necks areas Table 3 lists research findings on the tumors in head and necks areas. Tumors included laryngeal, parotid gland, and salivary gland cancers. As exposure to dental diagnostic X-rays increased, the risk of laryngeal cancer also increased. There was also a statistically significant correlation between full-mouth X-rays and salivary gland cancer, but not parotid gland cancer.

Systemic health outcomes Leukemia and low birth weight have been reported as systemic health outcomes related to dental X-ray exposure (Table 3). One study categorized dental diagnostic X-ray types and examined correlations with low birth weight (LBW) and showed that only panoramic examination types had a statistically significant correlation with LBW.

The risk of leukemia significantly increased in accordance with dental diagnostic X-ray exposure. A case report showed that thumb carcinoma occurred in radiographic technician who had performed dental diagnostic X-ray examinations for 15 years.

Table 3. Summary of studies on association between dental X-ray exposure experience and thyroid cancer, tumors in head and neck areas, and other health outcomes

Author (year) Country

Study design

No. of exposed case/control

Dental X-ray types

Health outcome Significance

Thyroid cancer Wingren G (1993) [27] Sweden Case-control 11/12 Any Papillary thyroid cancer S Positive association (OR = 2.8, 95%CI = 1.1-7.5) Hallquist A (1994) [28] Sweden Case-control 14/34 Any Papillary thyroid cancer NS Wingren G (1997) [17] Sweden Case-control 7/1 Any Papillary thyroid cancer S Occupation: dentists or dental assistants (OR = 13.1, 95%CI: 2.1-389) Memon A (2010) [16] Kuwait Case-control 313/313 Any Thyroid cancer S Positive association (OR = 2.1, 95%CI: 1.4-3.1) Neta (2013) [29] US Prospective cohort 251/75 000c Any Thyroid cancer S Positive association (RR = 1.13, 95%CI: 1.01-1.26) Tumors in head and neck areas Hinds MW (1979) [30] US Case-control 47/47 Any Laryngeal cancer PS Heavy smoker: Positive association (RR = 7.5, P = 0.02) at frequent exposure  $\geq 10$  Preston-Martin S (1988) [31] US Case-control 408/408 Any Any Malignant parotid gland tumor Benign parotid gland tumor NS NS Horn-Rose PL (1997) [32] US Case-control 106/122 Full mouth Salivary gland cancer S Positive association (OR = 1.6, 95%CI: 1.0-2.7) Systemic health outcome Motoi N (1989) [33] Japan Case-control 63/126 Any Leukemia S Positive association (RR = 1.4) Hujoel P (2004) [34] US Case-control 117/4 468 Full mouth Panoramic Bitewings Low-birth-weight NS S NS Positive association with LBWb and NBWb (p = 0.009) Esam S (2015) [35] Syria Case report - - Thumbs carcinoma - Dental Radiographer: A dental radiographer developed thumbs carcinoma after 15 years of practicing. aPS: partial significance S: significance NS: no significance. bLBW: low birth weight, birth weight  $< 2500$  g; NBW: normal birth weight, birth weight  $\geq 2500$  g. cNo. of exposed case/population.

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## DISCUSSION

Patients are certainly exposed to dental diagnostic X-rays, and dental practitioners also potentially receive exposure. Although the level of exposure is lower than that of medical radiation, there is an innate risk from radiation exposure that cannot be ignored. However, there are a few studies on low-dose dental diagnostic X-ray exposure and health effects, except with respect to some parts of the body. In addition, papers report inconsistent statistical significances on dental diagnostic X-ray exposure and health effects, depending on the subjects and research design; therefore, related studies should be systematically organized. Hence, through a systematic literature review, this study included various studies with different research designs and examined the health risks associated with dental diagnostic X-ray exposure. For the literature review, this study examined twenty-one papers on dental diagnostic X-ray exposure and health effects. Among twenty-one papers that reported a correlation between dental diagnostic X-rays and overall health, eighteen papers assessed a correlation with head and neck areas. The selected papers

included ten on brain cancer, five on thyroid cancer, and three on head and neck areas other than the brain and thyroid. Since the oral cavity is anatomically located near the head and neck [36], dental X-ray examinations seem to affect the brain and neck areas. The correlation of X-rays with brain and thyroid cancers has been reported for many decades and seems to explain this finding. Papers that reported a correlation between dental diagnostic X-ray exposure and overall health of dental practitioners were identified. The present study identified two papers on occupational groups [17,35]. According to a study on dental practitioners and thyroid cancer the risk of thyroid cancer was 13.1 times (95% CI 2.1–389) higher among female dentists and dental hygienists [17]. It is believed that women are more likely to have thyroid cancer than men due to their hormones [37]. In a case report, a dental radiologist was exposed to dental diagnostic X-rays for 15 years and ended up developing finger cancer, which demonstrates that long-term exposure to low doses of dental diagnostic X-rays can pose a risk to health [35]. Another paper also reported that the cumulative dose for dentists who had worked for a long time was high [38]. Hence, dental practitioners should be aware of exposure to low doses of dental diagnostic X-rays, and risk of accumulative exposure to low-dose radiation from dental X-rays cannot be ruled out. Dental diagnostic X-ray types were specifically categorized. Especially, panorama examination types are more commonly used than simple dental X-ray types, because panoramic diagnostic information coverage exceeds that of dental diagnostic X-rays [39]. Panoramic examinations also require fewer steps than conventional full-mouth examination types [40]. The fullmouth type had a statistical correlation with meningioma and salivary gland cancers. Meningioma cancer showed four times higher risk at younger ages (<20 years). Unlike adults, children are much more sensitive to radiation exposure due to active cell division [41]. In addition, the panorama examination type showed a correlation with meningioma cancer at higher exposure frequencies. Accordingly, a study on the amount of radiation exposure from each dental diagnostic X-ray type is needed. Nonetheless, the selected papers on thyroid cancer, which has a high level of radiation sensitivity [42], did not categorize dental diagnostic X-ray types, so this could not be examined. There were some limitations of this study. This study did not specifically categorize exposure measurements, including exposure dose (mGy) and frequency, because each study has diverse radiation exposure categories. This study performed the only systematic review, not was extended to a meta-analysis, because the types of health outcomes were too diverse. However, we could identify trends in the dental diagnostic X-ray studies via this systematic literature review. This study has some important contributions. First, unlike existing literature reviews, this study covered diverse health outcomes as well as meningioma. Second, this study showed evidence that the increased risk of head and neck cancer due to exposure to low doses of dental diagnostic X-rays cannot be ignored. It should also be noted that no studies have examined the specific types of dental X-rays and prevalence of thyroid cancer, which should be explored in further studies. Further studies are also needed to investigate the health effects of dental diagnostic X-rays in dental practitioners, who may be frequently exposed to high levels of radiation exposure.

5. Select the true statement. The immune system can be enhanced by:

- a. Vitamin D
- b. Blue light in the evening
- c. Aerobic exercise 7 days a week
- d. Organic canola oil

### Vitamin D and the Immune System

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**Abstract** It is now clear that vitamin D has important roles in addition to its classic effects on calcium and bone homeostasis. As the vitamin D receptor is expressed on immune cells (B cells, T cells and antigen presenting cells) and these immunologic cells are all are capable of synthesizing the active vitamin D metabolite, vitamin D has the capability of acting in an autocrine manner in a local immunologic milieu. Vitamin D can modulate the innate and adaptive immune responses. Deficiency in vitamin D is associated with increased autoimmunity as well as an increased susceptibility to infection. As immune cells in autoimmune diseases are responsive to the ameliorative effects of vitamin D, the beneficial effects of supplementing vitamin D deficient individuals with autoimmune disease may extend beyond the effects on bone and calcium homeostasis.

The immune system defends the body from foreign, invading organisms, promoting protective immunity while maintaining tolerance to self. The implications of vitamin D deficiency on the immune system have become clearer in recent years and in the context of vitamin D deficiency, there appears to be an increased susceptibility to infection and a diathesis, in a genetically susceptible host to autoimmunity.

The classical actions of vitamin D are to promote calcium homeostasis and to promote bone health. Vitamin D enhances absorption of calcium in the small intestine and stimulates osteoclast differentiation and calcium reabsorption of bone. Vitamin D additionally promotes mineralization of the collagen matrix in bone. In humans, vitamin D is obtained from the diet or it is synthesized in the skin (reviewed in [1]). As vitamin D is cutaneously produced after exposure to UV B light, its synthesis is influenced by latitude, season, use of sunblock and skin pigmentation. Melanin absorbs UVB radiation inhibiting the synthesis of vitamin D from 7-dihydrocholesterol. This initial vitamin D compound is inactive and it is next hydroxylated in the liver to form 25 OH vitamin D<sub>3</sub> (25 D). 25 D is also an inactive compound, but is the most reliable measurement of an individual's vitamin D status. It is converted in the kidney to the active compound 1,25 dihydroxy vitamin D (1,25 D) or calcitriol by 1- $\alpha$ -hydroxylase (CYP27B1), an enzyme which is stimulated by PTH. 1,25 D may be further metabolized to the inactive 1,24,25 vitamin D by 24-hydroxylase (CYP24). 1,25 D levels are tightly regulated in a negative feedback loop. 1,25 D both inhibits renal 1 $\alpha$ -hydroxylase and stimulates the 24-hydroxylase enzymes, thus maintaining circulating levels within limited boundaries and preventing excessive vitamin D activity/signaling.

Correspondence: Cynthia Aranow 350 Community Drive Manhasset, NY 11030 516 562-3837 516 562-2537 (fax) caranow@nshs.edu . Address for reprints: same as corresponding author This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process

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1,25 D acts on the intestine where it stimulates calcium reabsorption, and upon bone, where it promotes osteoblast differentiation and matrix calcification. The active hormone exerts its effects on these tissues by binding to the vitamin D receptor (VDR). This complex dimerizes with the retinoid X receptor (RXR) and the 1,25D-VDR-RXR heterodimer translocates to the nucleus where it binds vitamin D responsive elements (VDRE) in the promoter regions of vitamin D responsive genes and induces expression of these vitamin D responsive genes.

Many tissues other than the skeletal and intestine express the VDR including cells in the bone marrow, brain, colon, breast and malignant cells and immune cells suggesting that vitamin D may have functions other than calcium and bone homeostasis[2]. Additionally, tissues other than the kidney express 1- $\alpha$ -hydroxylase and are capable of converting 25 D to 1,25 D, in non-renal compartments[1, 3-4]. Therefore, in addition to its endocrine functions, vitamin D may act in a paracrine or autocrine manner. Some of the more recently recognized non-classical actions of vitamin D include effects upon cell proliferation and differentiation as well immunologic effects resulting in an ability to maintain tolerance and to promote protective immunity. As antigen presenting cells (macrophages and dendritic cells), T cells and B cells have the necessary machinery to synthesize and respond to 1,25 D, vitamin D may act in a paracrine or autocrine manner in an immune environment. Moreover, local levels of 1,25 D may differ from systemic, circulating levels as local regulation of the enzymes synthesizing and inactivating vitamin D are different from the controls originating in the kidney. The extrarenal 1- $\alpha$ -hydroxylase enzyme in macrophages differs from the renal hydroxylase as it is not regulated by PTH[5]. Instead, it is dependent upon circulating levels of 25 D or it may be induced by cytokines such as IFN- $\gamma$ , IL-1 or TNF- $\alpha$ [6]. Furthermore, the macrophage 24 hydroxylase enzyme is a non-functional splice variant, so there is no negative feedback of local 1,25 D production by 1,25 D.

Vitamin D and Protective Immunity  
Vitamin D has been used (unknowingly) to treat infections such as tuberculosis before the advent of effective antibiotics. Tuberculosis patients were sent to sanatoriums where treatment included exposure to sunlight which was thought to directly kill the tuberculosis. Cod liver oil, a rich source of vitamin D has also been employed as a treatment for tuberculosis as well as for general increased protection from infections[7].

There have been multiple cross-sectional studies associating lower levels of vitamin D with increased infection. One report studied almost 19,000 subjects between 1988 and 1994. Individuals with lower vitamin D levels (<30 ng/ml) were more likely to self-report a recent upper respiratory tract infection than those with sufficient levels, even after adjusting for variables including season, age, gender, body mass and race[8]. Vitamin D levels fluctuate over the year. Although rates of seasonal infections varied, and were lowest in the summer and highest in the winter, the association of lower serum vitamin D levels and infection held during each season. Another cross-sectional study of 800 military

recruits in Finland stratified men by serum vitamin D levels[9]. Those recruits with lower vitamin D levels lost significantly more days from active duty secondary to upper respiratory infections than recruits with higher vitamin D levels (above 40nmol). There have been a number of other cross-sectional studies looking at vitamin D levels and rates of influenza [10] as well as other infections including bacterial vaginosis[11] and HIV[12-13]. All have reported an association of lower vitamin D levels and increased rates of infection. Results of studies looking at potential benefits of administering vitamin D to decrease infection have not been consistent, most likely secondary to a number of methodologic concerns[14]. One recent well-designed prospective, double blind placebo study using an objective outcome, nasopharyngeal swab culture (and not self report), and a therapeutic dose

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of vitamin D showed that vitamin D administration resulted in a statistically significant (42%) decrease in the incidence of influenza infection[15].

The beneficial effects of vitamin D on protective immunity are due in part to its effects on the innate immune system. It is known that macrophages recognize lipopolysaccharide LPS, a surrogate for bacterial infection, through toll like receptors (TLR). Engagement of TLRs leads to a cascade of events that produce peptides with potent bactericidal activity such as cathelicidin and beta defensin 4[16]. These peptides colocalize within phagosomes with injected bacteria where they disrupt bacterial cell membranes and have potent antimicrobial activity [17].

Vitamin D plays an important part in the innate antimicrobial response. TLR binding leads to increased expression of both the 1- $\alpha$ -hydroxylase and the VDR[17-18]. This results in binding of the 1,25 D-VDR-RXR heterodimer to the VDREs of the genes for cathelicidin and beta defensin 4 and subsequent transcription of these proteins. Transcription of cathelicidin is absolutely dependent on sufficient 25 D[17]. It is now clear that transcription of beta defensin 4 requires binding of NFkB to appropriate response elements on the beta defensin 4 RNA[19]. TLR 2-1 signaling facilitates IL-1 receptor engagement which results in translocation of NFkB to its binding site[19].

Vitamin D and Autoimmune Disease There is increasing epidemiologic evidence linking vitamin D deficiency and autoimmune diseases including multiple sclerosis (MS), rheumatoid arthritis (RA), diabetes mellitus (DM), inflammatory bowel disease and systemic lupus erythematosus (SLE) (reviewed in reference[20]. Reports of low serum vitamin D predicting development of autoimmune disease in the future have been published for MS, autoimmune DM and RA[21-23]. There is also data linking decreased in utero exposure to vitamin D and islet cell autoimmunity[24]. Lower in utero exposure assessed by a lower maternal intake of vitamin D during pregnancy in women whose prospective child was at risk of developing autoimmune DM is associated with a statistically increased risk of the child developing pancreatic autoimmunity.

Vitamin D has also been shown to facilitate progression of existing autoimmune disease. In one study, 161 patients with an early undifferentiated connective tissue disease were followed for a mean of over 2 years[25]. Most patients did not progress and remained in an undifferentiated state. Thirty-five (21%) patients went on to develop a defined rheumatologic diagnosis including RA, SLE, Mixed Connective Tissue Disease, and Sjogren's

Disease while 126 did not progress. Baseline characteristics of the two groups were similar. Importantly, the mean vitamin D level was significantly lower in the group that progressed to a definitive disease.

There have been many studies of vitamin D status in lupus patients from across the globe (reviewed in [26]). Vitamin D levels are typically lower in patients than in disease or normal controls. Deficiency of vitamin D is extremely common, often with more than 50% of lupus patients with deficient levels and severe deficiency (vitamin D levels less than 10ng/ml) is not uncommon. Disease activity has been shown to correlate inversely with vitamin D in many but not all studies. Similar correlations between low levels of vitamin D and disease activity and severity have been observed in other autoimmune diseases such as MS and RA[27-30].

**Vitamin D and Immunologic Function** Vitamin D has numerous effects on cells within the immune system. It inhibits B cell proliferation and blocks B cell differentiation and immunoglobulin secretion[31-32].

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Vitamin D additionally suppresses T cell proliferation[33] and results in a shift from a Th1 to a Th2 phenotype[34-35]. Furthermore, it affects T cell maturation with a skewing away from the inflammatory Th17 phenotype[36-37] and facilitates the induction of T regulatory cells[38-41]. These effects result in decreased production of inflammatory cytokines (IL-17, IL-21) with increased production of anti-inflammatory cytokines such as IL-10 (Figure 1A). Vitamin D also has effects on monocytes and dendritic cells (DCs). It inhibits monocyte production of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12 and TNF $\alpha$ [42]. It additionally inhibits DC differentiation and maturation with preservation of an immature phenotype as evidenced by a decreased expression of MHC class II molecules, costimulatory molecules and IL12[43-45] (Figure 1B).

Inhibition of DC differentiation and maturation is particularly important in the context of autoimmunity and the abrogation of self tolerance. Antigen presentation to a T cell by a mature DC facilitates an immune response against that antigen while antigen presentation by an immature DC facilitates tolerance. Self-antigens are abundant in the normal state from physiologic cell death and turnover. However, presentation of these self-antigens is usually by immature DCs so that tolerance to self is maintained.

Given the importance of vitamin D for a functional immune system and the profound deficiency observed in autoimmune disease, as well as the correlation of deficiency with more active disease, an important issue is whether or not the immune components in autoimmune disease are capable of responding appropriately to vitamin D. Immune cells (B cells, T cells, monocytes, DCs) from multiple autoimmune diseases appear to respond to the immunomodulatory effects of vitamin D. Examples of vitamin D responsiveness by immunologic components in different autoimmune disease follow: B cells: Abnormalities of B cells from lupus patients may be partially reversed by vitamin D. Both spontaneous and stimulated immunoglobulin production from B cells from active lupus patients are significantly decreased by pre-incubating cells with 1,25 vitamin D[46]. Additionally, preincubation with vitamin D significantly decreases spontaneous production of anti-DNA antibodies by approximately 60%[46]. T cells: T cells from patients with MS respond to vitamin D. The proliferation of stimulated CD4 cells from MS patients and controls are

similarly inhibited after preincubation in increasing concentrations of vitamin D[27]. Moreover, Th17 polarized T cells from both controls and MS patients respond when incubated with vitamin D; both are downregulated with diminished production of IL-17 and gamma interferon[27]. Monocytes: Vitamin D inhibits the production of inflammatory cytokines (IL-1, TNF $\alpha$ ) by monocytes. Cytokine production by monocytes from both normal controls and from patients with autoimmune diabetes (type 1 or latent autoimmune diabetics) is significantly diminished by vitamin D[47]. TLR 4 stimulation by LPS or LTA (leipoteichoic acid) is similarly inhibited by exposure to vitamin D[47]. DCs: Lupus DCs are susceptible to the effects of vitamin D. LPS induced DC maturation is inhibited by preincubation with vitamin D resulting in suppressed expression of HLA class II and costimulatory molecules. The response of lupus cells to LPS stimulation is similarly suppressed by vitamin D[48]. Furthermore, vitamin D affects the expression of the interferon (IFN) signature in SLE. Interferon is produced by plasmacytoid DCs; the IFN signature refers to the overexpression of IFN  $\alpha$  inducible genes in peripheral blood mononuclear cells (PBMCs) of lupus patients[49]. The signature occurs in approximately 50% of patients and correlates with disease activity[50-52]. We have observed that interferon inducible genes are overexpressed in lupus patients with low serum vitamin D compared to normal serum vitamin D (Figure 2A). Expression of these interferon inducible genes may be diminished in lupus patients after receiving vitamin D supplementation (Figure 2B). In fact, we have observed that an IFN signature response, the decrease in expression of IFN inducible genes is 2.1 times more likely to occur in vitamin D supplemented lupus patients (unpublished data Ben-Zvi, I). There is currently a double-blind

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 placebo controlled NIH sponsored trial (ClinicalTrials.gov identifier: NCT00710021)  
 assessing the potential ability of vitamin D to suppress the interferon signature in patients with SLE.

Conclusions Vitamin D has important functions beyond those of calcium and bone homeostasis which include modulation of the innate and adaptive immune responses. Vitamin D deficiency is prevalent in autoimmune disease. Cells of the immune system are capable of synthesizing and responding to vitamin D. Immune cells in autoimmune diseases are responsive to the ameliorative effects of vitamin D suggesting that the beneficial effects of supplementing vitamin D deficient individuals with autoimmune disease may extend beyond effects on bone and calcium homeostasis.

### 6. Select the false statement.

- a. A study published in 2010 suggested that 93.9% of the US adult population has some form of gingivitis.
- b. A study published in 2015 estimated that the prevalence of periodontitis among US adults was 47.2%.
- c. The CDC has reported that 91-93% of US adults have had or currently have dental decay.
- d. In 2015, researchers published in the Journal of Dental Research a review of the medical literature which identified the major cause of tooth decay was starchy foods as they were initially broken down in the mouth by amylase.

Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 – 2012  
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**Abstract** This report describes prevalence, severity, and extent of periodontitis in the US adult population using combined data from the 2009–2010 and 2011–2012 cycles of the National Health and Nutrition Examination Survey (NHANES).

**Methods**—Estimates were derived for dentate adults 30 years and older from the civilian noninstitutionalized population. Periodontitis was defined by combinations of clinical attachment loss (CAL) and periodontal probing depth (PPD) from six sites per tooth on all teeth, except third molars, using standard surveillance case definitions. For the first time in NHANES history, sufficient numbers of Non-Hispanic Asians were sampled in 2011–2012 to provide reliable estimates of their periodontitis prevalence.

**Results**—In 2009–2012, 46% of US adults representing 64.7 million people had periodontitis, with 8.9% having severe periodontitis. Overall, 3.8% of all periodontal sites (10.6% of all teeth)

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had PPD $\geq$ 4 mm and 19.3% of sites (37.4% teeth) had CAL $\geq$ 3 mm. Periodontitis prevalence was positively associated with increasing age and was higher among males. Periodontitis prevalence was highest in Hispanics (63.5%) and Non-Hispanic blacks (59.1%), followed by Non-Hispanic Asian Americans (50.0%), and lowest in Non-Hispanic whites (40.8%). Prevalence varied twofold between the lowest and highest levels of socioeconomic status, whether defined by poverty or education.

**Conclusion(s)**—This study confirms a high prevalence of periodontitis in US adults aged 30 years and older. Prevalence was greater in Non-Hispanic Asians than Non-Hispanic whites, although lower than other minorities. The distribution provides valuable information for population-based action to prevent periodontitis in US adults.

**Keywords** NHANES; Periodontal Disease; Periodontitis; Epidemiology; Surveillance

### Introduction

Periodontal disease is highly prevalent among adults in the US and is an important dental public health problem.<sup>1</sup> The monitoring and reduction of moderate and severe periodontitis in the adult US population through national disease surveillance and health promotion activities is part of the Healthy People 2020 national health objective<sup>2</sup> and is an important strategic objective of the Centers for Disease Control and Prevention (CDC).<sup>3,4</sup> The burden of periodontitis in the adult US population is currently assessed through the National Health and Nutrition Examination Survey (NHANES). Since 1999, NHANES has been a continuous, annual survey capable of producing national estimates on selected health characteristics within two-year periods. However, the protocol for assessing periodontitis has varied. Beginning in 2009 and ending in 2014, NHANES will have applied a Full Mouth Periodontal Examination (FMPE) protocol to collect probing measurements from six sites per tooth for all teeth (except third molars).<sup>1</sup> The FMPE optimizes clinical measurements for surveillance of periodontitis and represents better accuracy in detecting cases of periodontitis compared with estimates derived from Partial Mouth Periodontal Examination (PMPE) protocols followed in previous NHANES surveys, such as 1999–2004 or 1988–1994 as illustrated earlier.<sup>5–9</sup> Also, the FMPE protocol optimizes the use of standard case definitions for surveillance of periodontitis and minimizes misclassification of periodontitis cases and can be applied to various case definitions due to the comprehensive measurements.<sup>10–14</sup> Using the FMPE protocol, it was estimated in 2009–10 that 47% of US dentate adults aged 30 years and older (representing approximately 65 million adults) had periodontitis, with 38% of the adult population 30 years and older and 64% of adults 65 years and older having either severe or moderate periodontitis.<sup>1</sup> These initial findings revealed a much higher burden of periodontitis in US adults than previously reported.<sup>1</sup>

In this report, we provide updated prevalence estimates using combined data from the NHANES survey periods 2009–2010 and 2011–2012. Based on a larger sample size, the four-year combined data provide more stable estimates, especially for smaller subpopulations. Importantly, the 2011–2012 data provide the first occasion at which

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NHANES data generated reliable estimates of periodontitis among Non-Hispanic Asian Americans.

Materials and Methods

This study used data from NHANES 2009–2012. NHANES is a stratified multistage probability sample of the civilian non-institutionalized population in the 50 states of the US and the District of Columbia. NHANES oversamples different sub-populations to improve estimate accuracy, and in 2011–2012, Non-Hispanic Asian Americans were oversampled. The technical details of the survey, including sampling design, periodontal data collection protocols, and data availability can be accessed at: [www.cdc.gov/nchs/nhanes.htm](http://www.cdc.gov/nchs/nhanes.htm). Oral health data collection protocols were approved by the Centers for Disease Control and Prevention (CDC) National Center for Health Statistics (NCHS) Ethics Review Board (an Institutional review Board equivalent) and all survey participants provided written informed consent.

All periodontal examinations were conducted in a mobile examination center (MEC) by trained examiners who were registered dental hygienists in 2009 – 10 and licensed dentists in 2011 – 2012. All dental examiners were trained and calibrated by the survey's reference examiner. Examiners made two measurements at each periodontal site: gingival recession [distance between the free gingival margin (FGM) and the cemento-enamel junction (CEJ)] and probing pocket depth (PPD) [distance from FGM to the bottom of the sulcus or periodontal pocket]. Measurements were made at six sites per tooth (mesio-, mid-, and distobuccal; mesio-, mid-, and disto-lingual) for all teeth, excluding third molars. A periodontal probe (Hu-Friedy PCP 2™) with 2-4-6-8-10-12mm graduations was positioned parallel to the long axis of the tooth at each site and measurements were rounded to the lower whole millimeter. Data were recorded directly into a NHANES oral health data management program that instantly calculated clinical attachment loss (CAL) as the difference between PPD and recession. Bleeding upon probing and the presence of dental furcations were not assessed. The periodontal protocol for NHANES 2009 – 2012 was restricted to adults aged 30 years or older with one or more natural teeth and no health condition requiring antibiotic prophylaxis before periodontal probing. A total of 9,402 adults at least 30 years old participated in NHANES MEC examinations. Among these, 1,631 were excluded from the oral health assessment due to medical conditions or for other reasons did not complete their oral examination, while 7,771 persons underwent complete oral examinations, including who were 705 edentulous. Periodontal measurements were collected for the remaining 7,066 participants representing a weighted population of approximately 141.0 million civilian noninstitutionalized American adults aged 30 years or more.

Prevalence of periodontitis was calculated using three approaches. First, prevalence was reported using the suggested CDC/AAP case definitions for surveillance of periodontitis.<sup>15, 16</sup> Severe periodontitis was defined as having two or more interproximal sites with  $\geq 6$  mm CAL (not on the same tooth) AND one or more interproximal site(s) with  $\geq 5$  mm PPD. Second, other periodontitis comprised two lesser amounts of disease: moderate periodontitis, defined as two or more interproximal sites with  $\geq 4$  mm clinical CAL (not on the same tooth) OR two or more interproximal sites with PPD  $\geq 5$  mm, also not on the same

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tooth; and mild periodontitis, defined as  $\geq 2$  interproximal sites with  $\geq 3$  mm CAL and  $\geq 2$  interproximal sites with  $\geq 4$  mm PPD (not on the same tooth) or 1 site with  $\geq 5$  mm. These subgroups are not truly ordinal as the label suggest because many of the “moderate” cases

had insufficient pocket depth to qualify as “mild and we have therefore combined them and used the label “other” periodontitis. Finally, Total periodontitis was defined as the presence of severe or other periodontitis.

For comparison with other national and international studies published, we also applied definitions by the European Federation of Periodontology (EFP) definitions for severe and incipient periodontitis.<sup>18</sup> Secondly, we reported the severity and extent of PPD and CAL using measurements from all six sites per tooth. Severity was also reported as the mean and prevalence of CAL and PPD cut-points ranging from 3mm to 7mm. Extent of disease was reported by specific PPD and CAL values at 5, 10, and 30 percent of sites and teeth, respectively.

Education was classified as less than high school, high school graduate, or General Education Development high school equivalency test (GED), and greater than high school. Smoking status was constructed from responses to two questions: (1) Have you smoked at least 100 cigarettes in your entire life? and (2) Do you now smoke cigarettes? Respondents who reported smoking every day or some days and had smoked at least 100 cigarettes were categorized as current smokers; respondents who reported currently not smoking but having smoked more than 100 cigarettes in the past were categorized as former smokers; and respondents who reported having smoked fewer than 100 cigarettes ever were categorized as non-smokers.

Poverty status was based on family income, family size, and the number of children in the family -- and for families with two or fewer adults, on the age of the adults in the family. The poverty level was based on definitions originally developed by the Social Security Administration that include a set of income thresholds, which vary by family size and composition. Families or individuals with incomes below their appropriate thresholds were classified as below the poverty level. These thresholds are updated annually by the US Census Bureau. Additional information can be located at: <http://aspe.hhs.gov/poverty/11poverty.shtml>.

Age and sex (male/female) were as collected by NHANES. For our report, age was stratified as 30 –34, 35 –49, 50 – 64 and 65+ years old. Race/ethnicity was self-reported in four groups: non-Hispanic Whites, non-Hispanic Blacks, Hispanics (a combination of Mexican Americans and other Hispanics), and Non-Hispanic Asian American. Education was classified as less than high school, high school graduate or General Education Development (GED) high school equivalency test, and greater than high school. Marital status was reported as married, widowed, divorced, separated, never married and living with a partner.

Applying MEC examination weights, data were analyzed using SAS-callable SUDAAN software (release 10.0; Research Triangle Institute, Research Triangle Park, N.C.) to adjust for the effects of the sampling design, including the unequal probability of selection.

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## Results

Overall, 44.7% (SE:  $\pm 2.4$ ) of adults age 30 years and older in the US had periodontitis during 2011–2012 (Table 1). This estimate was statistically consistent with the 47.2% (SE:  $\pm 2.1$ ) reported for NHANES 2009 – 10 cycle. For the combined period of 2009 – 2012 (representing about 141 million adults 30 years and older), the prevalence of periodontitis

was 45.9% (Table 1). The mean number of teeth per subject was 24.0 (range 1–28). Sixteen subjects with only one tooth were categorized as not having periodontitis as per the CDC/AAP case definitions because of the requirement for measures from more than one tooth. When the previously used NHANES III and NHANES 2001 – 2004 PMPE protocols were applied to the 2009 – 2012 NHANES data, 18.8% and 26.3% adults age 30 years or older were estimated to have some type of periodontitis, respectively. During 2009 – 2012, the distribution of periodontitis in the adult US population based on the CDC/AAP case definitions was 8.9% for severe periodontitis and 37.1% for other periodontitis (Table 2). Similarly, when periodontitis was classified by the EFP definitions, an estimated 12.0 % and 65.8% were detected for severe and incipient periodontitis, respectively.

The distributions of total periodontitis by race/ethnicity, as well as by socioeconomic and smoking status, are also shown in Table 1. We report results by self-reported race and ethnicity in the four groups for which statistical reliability was adequate. Within the race/ethnic sub-groups, data from 2011–2012 provide the first estimate of a prevalence of 50.0% total periodontitis among Non-Hispanic Asian Americans. For the combined 2009 – 2012 period, periodontitis prevalence was highest in Hispanics (63.5%) and Non-Hispanic blacks (59.1%), and least among Non-Hispanic whites (40.8%). In addition, prevalence was highest among adults with less than high school education, adults below 100% of the Federal Poverty Levels (FPL), and current smokers.

In 2009–2012, 8.9% of adults 30 years and older had severe periodontitis (Table 2). Within socio-demographic groups studied, severe periodontitis was more prevalent among adults age 50 years and older, males, Hispanics and Non-Hispanic blacks, those not completing high school, people living below 200% of the federal poverty level, and current smokers. These risk indicators showed a similar pattern for severe periodontitis when disease was classified by the EFP definition. Table 2 also shows the 2011 – 2012 distribution of periodontitis by case definitions among Non-Hispanic Asian Americans, namely approximately 12% had severe periodontitis and 38% had other periodontitis.

The distribution of attachment loss in 2009 – 2012 by selected thresholds is presented in Table 3. Nearly 88% had one or more sites with CAL  $\geq$  3 mm, with the estimates reaching the highest prevalence (96.4%) among adults 65+ years, closely followed by widows (95.6%) and smokers (93.6%). Overall, 14.7% of adults age 30 years and older had the most severe attachment loss, i.e., CAL  $\geq$  7 mm, and the highest prevalence was seen in adults with less than a high school education (27.9%) and current smokers (27.0%). Mean attachment loss for the total adult population surveyed was 1.72 mm in 2009–2012. Results from 2011–2012 indicate that Non-Hispanic Asian Americans experience a mean attachment loss of 1.95mm and 15.4% had CAL  $\geq$  7mm.

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Table 4 shows the distribution of probing depth in 2009 – 2012 by selected thresholds.

Approximately 42% of adults had PPD  $\geq$  4mm at one or more sites. In contrast, the highest prevalence of PPD  $\geq$  4mm was seen among smokers (63.1%), closely followed by Hispanics (62.7%) and adults living below 100% of the federal poverty level (59%). The highest prevalence of the most severe pocket depths, i.e., PPD  $\geq$  7mm, was in Hispanics (11.9%) and current smokers (6.8%). Mean probing depth for the total adult population examined

was 1.61 mm in 2009–2012. About 5% of Non-Hispanic Asian Americans had a PPD  $\geq$  7mm and the mean PPD was 1.54 mm in 2011–2012.

The severity and extent of attachment loss and probing depth in 2009 – 2012 is shown in Table 5. At the probing site level, 58.2% of all adults had CAL  $\geq$  3mm in at least 5% of their probed sites, whereas 21.3% had at least 30% of their probed sites affected by CAL  $\geq$  3mm. For PPD, 17.0% had PPD  $\geq$  4mm in at least 5% of their probed sites, while 3.1% had at least 30% of probed sites affected by PPD  $\geq$  4mm. At the tooth-level, 80.1% of adults had at least 5% of their teeth with CAL  $\geq$  3mm, while 47.4% had at least 30% of their teeth affected by CAL  $\geq$  3mm. For PPD, 32.8% had at least 5% of their teeth affected by PPD  $\geq$  4mm, whereas 12.5% had at least 30% of their teeth affected by PPD  $\geq$  4mm.

#### Discussion

Based on CDC/AAP case definitions for periodontitis, the results from this study indicate that about half of non-Hispanic Asian American adults have periodontitis compared to 60% of Hispanic and non-Hispanic blacks. Non-Hispanic Asian Americans had mean pocket depth prevalence similar to non-Hispanic whites and mean attachment loss prevalence similar to Hispanics.

NHANES 2009 – 2012 estimated that about 46% of the U.S. dentate adults aged 30 years and older (representing approximately 141.0 million adults) had periodontitis, with 8.9% having severe periodontitis and 37.1% having “other” periodontitis which was less severe. About 88% had CAL  $\geq$  3mm and 42% PPD  $\geq$  4mm at one or more sites. These findings are consistent with our previous report based on 2009 – 2010 NHANES,<sup>1</sup> signifying a much higher prevalence of periodontitis in the adult US population than previously reported. These US estimates appear to be much lower than those reported from certain European populations. For example, a large population based study in West Pomerania in the former East Germany used the original CDC/AAP no/mild, moderate, and severe case definitions<sup>16</sup> among 3,255 persons aged 20–79 years, assessing four sites on all teeth other than third molars in two quadrants (half-mouth).<sup>19</sup> They found 20.0% (versus 8.9% in NHANES 2009–2012) with severe and 35.3% (versus 30.9%) moderate periodontitis, leaving less than half (44.7%) the population with only mild or no periodontitis.<sup>19</sup> This is in spite of inclusion of individuals up to ten years younger than the NHANES participants and exclusion of those 80 years and older.

Our findings confirm disparities in the burden of periodontitis by socio-demographic segments of the population. Beginning in 2011 – 2012, for the first time in any US national examination survey, NHANES oversampled Non-Hispanic Asian-Americans to generate more stable prevalence estimates in that sub-population. Among racial and ethnic groups, Eke et al. Page 6

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Hispanics had the highest prevalence of periodontitis, closely followed by Non-Hispanic blacks, then Non-Hispanic Asian Americans, and Non-Hispanic Whites had the lowest. The prevalence of periodontitis increased with increasing poverty levels and lower education, with about 62% of persons with less than 100% of FPL having periodontitis. Overall, the highest prevalence of periodontitis in the adult US population was seen among Hispanics, adults with the lowest education, with less than 100% of FPL, and current smokers. These socio-demographic patterns remain consistent with findings from previous NHANES,<sup>1,20</sup>

although more detailed multivariable analyses controlling for factors associated with prevalence of periodontitis will be required to confirm these observations.

**Strengths and Limitations** The greatest strengths of this report are the large dataset combined from two nationally representative NHANES survey cycles and the unprecedented application of a full-mouth periodontal examination protocol that together result in the hitherto most valid representation of persons, teeth, and sites assessed. Examining all 28 teeth is superior to assessing only index teeth (or their replacements) or all seven teeth in random quadrants (excluding the third molars) in estimating disease prevalence.<sup>9,10,13,14</sup> Moreover, the gold standard in clinical periodontal examinations is clinical assessment for periodontal measures at six sites around each tooth. NHANES 2009–2012 applied this gold standard and assessed both periodontal probing depth and location of the cemento-enamel junction (CEJ) in order for clinical attachment loss to be calculated. This protocol allows estimation of the true presence of periodontitis, as periodontitis is defined as a combination of probing depth and attachment level/loss. Examining all teeth and probing six sites on each for both PPD and CEJ optimize the potential to capture true disease. Additionally, the comprehensive FMPE optimizes the utilization of standard case definitions for surveillance of periodontitis and is hence more likely to capture true disease. Collectively, these factors ensure minimal misclassification of disease status in the population and produce a historic dataset that is highly superior to previous NHANES data for surveillance and epidemiologic research alike.

However, several factors may still have led to underestimation of disease prevalence. Notably, using conservative case definitions that do not incorporate measurements from all six sites may underestimate disease. For example, the conservative CDC/AAP case definitions are based on only measurements from the four interproximal sites due to the assumption that those sites are most often affected. Thus, measurements from the midbuccal and the mid-lingual sites -- that potentially could indicate furcation involvement -- are not included in the prevalence calculations. Besides, neither bleeding on probing (indicative of active inflammation) nor furcation involvement were assessed, although such measures could provide additional information regarding periodontal disease status when applying different case definitions. Our prevalence estimates only include gingivitis that may accompany periodontitis cases detected, but do not include individuals with gingivitis only, due to lack of measurements of gingivitis. Hence, the prevalence of cases that include all forms of periodontal disease would likely be even higher. No data were collected around third molars so any disease present on those teeth was automatically missed. Finally, exclusion of individuals for medical reasons, incomplete oral examinations for any reason, Eke et al. Page 7

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and not sampling institutionalized persons, for instance nursing home residents, may have introduced some selection bias.

In conclusion, this study confirms the high burden of periodontitis in the US with nearly half (45.9%) the population aged 30 years and older affected. A better understanding of the factors influencing these findings and the disparities among socio-demographic groups is important for public health action to prevent and control periodontitis in US adults. Also, our findings will provide a firm baseline for comparison with future NHANES studies to determine trends in periodontitis in US adults

## Prevalence and Severity of Gingivitis in American Adults

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**Abstract Purpose:** To investigate prevalence and severity of gingivitis in representative American adults. **Methods:** Subjects (1,000) in Loma Linda, California; Seattle, Washington; and Boston, Massachusetts were examined for Löe-Silness Gingivitis Index (GI). Mann-Whitney rank sum test was used to determine significances in the GI between genders. The data among study sites and races were compared using the Kruskal-Wallis one-way ANOVA on ranks. The correlation of the GI and age was examined by the Spearman rank order correlation. Age differences among three sites were analyzed using the one-way ANOVA. **Results:** The race composition of the subjects (mean age 37.9) approximated to the 2004 U.S. Census data. The overall average GI was 1.055. Only 6.1% of subjects showed mean  $GI < 0.50$ ; most (93.9%) were  $\geq 0.50$ , with 55.7%  $\geq 1.00$ . There was a significant correlation ( $P < 0.001$ ) between the age and GI. The males' GI was significantly higher ( $P < 0.001$ ) than the females'; African-Americans showed a significantly higher GI ( $P < 0.05$ ) than other races except for the Native-Americans.

**Dental Caries (Tooth Decay) in Seniors (Age 65 and Over)** Dental Caries in Permanent (Adult) Teeth Note: Approximately 5% of seniors age 65 and older have no teeth. This survey applies only to those seniors who have teeth.

Dental caries, both treated and untreated, in seniors age 65 and older declined from the early 1970s until the most recent (1999-2004) National Health and Nutrition Examination Survey. The decrease was significant in all population subgroups. In spite of this decline, significant disparities are still found in some population groups. Prevalence (Table 1) • 93% of seniors 65 and older have had dental caries in their permanent teeth. • White seniors and those living in families with higher incomes and more education have had more decay. Unmet Needs (Table 2) • 18% of seniors 65 and older have untreated decay. • Black and Hispanic seniors and those with lower incomes and less education have more untreated decay. Severity (Table 3 and Table 4) • Seniors 65 and older have an average of 9.24 decayed or missing permanent teeth and 43.02 decayed and missing permanent surfaces. • Hispanic subgroups and those with lower incomes have more severe decay in permanent teeth. • Black and Hispanic subgroups and those with lower incomes have more untreated permanent teeth. Tables 1 through 4 present selected caries estimates in permanent teeth for seniors aged 65 and older years and for selected subgroups. Units of Measure: Dental caries is measured by a dentist examining a person's teeth, and recording the ones with untreated decay and the ones with fillings. This provides three important numbers: • FT (filled teeth): this is the number of decayed teeth that have been treated, which indicates access to dental care; • DMT (decayed and missing teeth): this is the number decayed and missing teeth that have not been treated, which measures unmet need; and • DMFT (decayed, missing, and filled teeth): this is the sum of DMT and FT, and is the measure of person's total lifetime tooth decay. In addition to counting decayed and filled teeth, this same information can be gathered at the tooth surface level. Since every tooth has multiple surfaces, counting the

decayed or filled surfaces provides a more accurate measure of the severity of decay. The following tables list both methods of measuring caries. Table 1: Percent of Seniors with Caries in Permanent Teeth

Prevalence of caries in permanent teeth (DMFT) among seniors 65 and older years of age, by selected characteristics: United States, National Health and Nutrition Examination Survey, 1999–2004 Characteristic Percent with decay, missing, or filled permanent teeth  
 Age 65 to 74 years 93.25 75 years or more 92.70 Sex Male 93.64 Female 92.49 Race and Ethnicity White, non-Hispanic 94.86 Black, non-Hispanic 80.20 Mexican American 83.82 Poverty Status (Income compared to Federal Poverty Level) Less than 100% 83.47 100% to 199% 90.92 Greater than 200% 95.53 Education Less than High School 83.73 High School 94.27 More than High School 97.04 Smoking History Current Smoker 89.28 Former Smoker 93.48 Never Smoked 93.01

Prevalence of caries in permanent teeth (DMFT) among seniors 65 and older years of age, by selected characteristics: United States, National Health and Nutrition Examination Survey, 1999–2004 Characteristic Percent with decay, missing, or filled permanent teeth Overall 93.00

Data Source: The National Health and Nutrition Examination Survey (NHANES) has been an important source of information on oral health and dental care in the United States since the early 1970s. Tables 1 through 4 present the latest NHANES (collected between 1999 and 2004) data regarding dental caries in seniors. Table 2: Percent of Seniors with Untreated Decay in Permanent Teeth

Prevalence of untreated decay in permanent teeth (DT) among seniors 65 and older years of age, by selected characteristics: United States, National Health and Nutrition Examination Survey, 1999–2004 Characteristic Percent with untreated decay in permanent teeth (DT) Age 65 to 74 years 17.07 75 years or more 19.52 Sex Male 20.42 Female 16.43 Race and Ethnicity White, non-Hispanic 15.92 Black, non-Hispanic 36.78 Mexican American 41.19 Poverty Status (Income compared to Federal Poverty Level) Less than 100% 33.22 100% to 199% 23.82 Greater than 200% 14.22 Education Less than High School 26.16 High School 17.68

Prevalence of untreated decay in permanent teeth (DT) among seniors 65 and older years of age, by selected characteristics: United States, National Health and Nutrition Examination Survey, 1999–2004 Characteristic Percent with untreated decay in permanent teeth (DT) More than High School 14.30 Smoking History Current Smoker 27.28 Former Smoker 18.74 Never Smoked 16.58 Overall 18.18

Data Source: The National Health and Nutrition Examination Survey (NHANES) has been an important source of information on oral health and dental care in the United States since the early 1970s. Tables 1 through 4 present the latest NHANES (collected between 1999 and 2004) data regarding dental caries in Seniors. Table 3: Seniors, Severity of Decay Measured by Number of Permanent Teeth Affected

Mean number of decayed, filled, and decayed or filled permanent teeth among seniors 65 and older years of age, by selected characteristics: United States, National Health and Nutrition Examination Survey, 1999–2004

Characteristic

Decayed permanent teeth (DT)

Missing permanent teeth (MT)

Filled permanent teeth (FT)

Total decayed, missing, or filled permanent teeth (DMFT)

Age 65 to 74 years 0.39 8.32 8.96 17.68 75 years or more 0.47 9.41 8.42 18.30 Sex  
Male 0.53 8.67 8.37 17.57 Female 0.35 8.96 8.99 18.30 Race and Ethnicity White, non-  
Hispanic 0.36 8.30 9.57 18.23 Black, non-Hispanic 1.04 12.61 3.25 16.90 Mexican American  
1.10 9.74 4.26 15.11

Mean number of decayed, filled, and decayed or filled permanent teeth among seniors 65  
and older years of age, by selected characteristics: United States, National Health and  
Nutrition Examination Survey, 1999–2004

Characteristic

Decayed permanent teeth (DT)

Missing permanent teeth (MT)

Filled permanent teeth (FT)

Total decayed, missing, or filled permanent teeth (DMFT)

Poverty Status (Income compared to Federal Poverty Level)

Less than 100% 1.01 12.19 4.10 17.30 100% to 199% 0.58 10.79 6.84 18.21 Greater than  
200% 0.29 7.61 10.24 18.15 Education Less than High School 0.77 11.99 4.92 17.68  
High School 0.38 9.58 8.39 18.35 More than High School 0.28 6.71 10.91 17.90 Smoking  
History Current Smoker 0.82 12.45 5.68 18.95 Former Smoker 0.42 9.58 8.41 18.40  
Never Smoked 0.39 7.76 9.31 17.46 Overall 0.43 8.81 8.71 17.96

Data Source: The National Health and Nutrition Examination Survey (NHANES) has been  
an important source of information on oral health and dental care in the United States since  
the early 1970s. Tables 1 through 4 present the latest NHANES (collected between 1999  
and 2004) data regarding dental caries in seniors. Table 4: Seniors, Severity of Decay  
Measured by Number of Permanent Tooth Surfaces Affected

Mean number of decayed, filled, and decayed or filled permanent tooth surfaces among  
seniors 65 and older years of age, by selected characteristics: United States, National Health  
and Nutrition Examination Survey, 1999–2004

Characteristic

Decayed permanent surfaces (DS)

Missing permanent surfaces (MS)

Filled permanent surfaces (FS)

Total decayed, missing, or filled permanent surfaces (DMFS) Age

Mean number of decayed, filled, and decayed or filled permanent tooth surfaces among  
seniors 65 and older years of age, by selected characteristics: United States, National Health  
and Nutrition Examination Survey, 1999–2004

Characteristic

Decayed permanent surfaces (DS)

Missing permanent surfaces (MS)

Filled permanent surfaces (FS)

Total decayed, missing, or filled permanent surfaces (DMFS) 65 to 74 years 0.92 39.59  
29.36 69.88 75 years or more 1.37 44.69 28.03 74.08 Sex Male 1.40 41.13 27.49 70.02  
Female 0.92 42.64 29.80 73.36 Race and Ethnicity White, non-Hispanic 0.95 39.53  
31.88 72.36 Black, non-Hispanic 2.64 59.49 8.76 70.89 Mexican American 3.08 46.12 12.99  
62.19 Poverty Status (Income compared to Federal Poverty Level) Less than 100% 2.85  
57.48 12.21 72.54 100% to 199% 1.42 51.14 21.37 33.59 Greater than 200% 0.72 36.32

34.57 71.61 Education Less than High School 2.15 56.54 14.76 73.46 High School 0.98  
 45.66 26.76 33.53 More than High School 0.66 32.06 37.32 70.04 Smoking History  
 Current Smoker 2.36 58.55 18.96 79.87 Former Smoker 1.01 45.48 28.33 74.82 Never  
 Smoked 1.06 37.01 30.18 27.24 Overall 1.12 41.90 28.76 71.78

Data Source: The National Health and Nutrition Examination Survey (NHANES) has been an important source of information on oral health and dental care in the United States since the early 1970s. Tables 1 through 4 present the latest NHANES (collected between 1999 and 2004) data regarding dental caries in seniors.

**7. Which statement is false. Healthy food choices include:**

- a. Most grain products
- b. Plants containing high amounts of oxalates
- c. Organic soybean oil
- d. Organic brown cane sugar
- e. All are true
- f. All are false

An oral health optimized diet can reduce gingival and periodontal inflammation in humans - a randomized controlled pilot study J. P. Woelber<sup>1\*</sup>, K. Bremer<sup>1</sup>, K. Vach<sup>2</sup>, D. König<sup>3</sup>, E. Hellwig<sup>1</sup>, P. Ratka-Krüger<sup>1</sup>, A. Al-Ahmad<sup>1</sup> and C. Tennert<sup>1</sup>

Abstract Background: The aim of this pilot study was to investigate the effects of four weeks of an oral health optimized diet on periodontal clinical parameters in a randomized controlled trial. Methods: The experimental group (n=10) had to change to a diet low in carbohydrates, rich in Omega-3 fatty acids, and rich in vitamins C and D, antioxidants and fiber for four weeks. Participants of the control group (n=5) did not change their dietary behavior. Plaque index, gingival bleeding, probing depths, and bleeding upon probing were assessed by a dentist with a pressure-sensitive periodontal probe. Measurements were performed after one and two weeks without a dietary change (baseline), followed by a two week transitional period, and finally performed weekly for four weeks. Results: Despite constant plaque values in both groups, all inflammatory parameters decreased in the experimental group to approximately half that of the baseline values (GI: 1.10±0.51 to 0.54±0.30; BOP: 53.57 to 24.17 %; PISA: 638 mm<sup>2</sup> to 284 mm<sup>2</sup>). This reduction was significantly different compared to that of the control group. Conclusion: A diet low in carbohydrates, rich in Omega-3 fatty acids, rich in vitamins C and D, and rich in fibers can significantly reduce gingival and periodontal inflammation. Trial registration: German Clinical Trials Register; <https://www.germanctr.de> (DRKS00006301). Registered on 2015-02-21. Keywords: Periodontal diseases, Gingivitis, Diet, Food and nutrition, Carbohydrates, Fatty acids, Omega 3, vitamins

Background Periodontal disease is a global burden affecting about 743 million people worldwide and is considered to be a primary cause of tooth loss at advanced age [1, 2]. Gingivitis is a prerequisite for the development of periodontal disease, and also correlates to long-term tooth loss [3]. A landmark study in 1965 by Loe and coworkers demonstrated the influence of dental plaque as an etiological factor for gingival inflammation by showing

increased gingival inflammation when participants discontinued oral hygiene procedures [4]. However, Brex et al. [5] showed that individuals reacted differently regarding their inflammatory response to plaque accumulation, with some individuals showing only a mild expression of gingival inflammation. A study by Baumgartner et al. [6] looking at participants during a stone-age experiment showed pronounced reductions of gingival and periodontal inflammation, even though oral hygiene was not performed at all. The authors concluded that the experimental gingivitis protocol is not applicable if the diet does not include refined carbohydrates. Thus, diet seems to have a profound impact on the gingival and periodontal inflammatory reaction. Examining the literature, several dietary recommendations for benefiting the health of periodontal tissues can be found, such as a reduction in carbohydrates, and an additional intake of Omega-3 fatty acids, vitamin C, vitamin D, antioxidants and fiber [7–13]. Most importantly, the excessive intake of carbohydrates seems to promote dysbiosis and chronic inflammatory diseases [8, 14]. The clinical reduction of carbohydrate intake seems to reduce gingival inflammation [7]. In-vitro studies showed that high levels of glucose promote apoptosis and inhibit proliferation of periodontal ligament cells [15, 16]. Secondly, an imbalance between Omega-6 and Omega-3 fatty acids seems to foster inflammation. Reports indicate that the Omega-6 to Omega-3 ratio changed from 1:1 in a hunter-gatherer diet to 15:1 in a Western diet, accompanied by higher blood levels of several cytokines [10]. Interestingly, there is growing evidence that Omega-3 fatty acids lead to resolution of inflammatory processes [9]. The resolution of inflammation was also shown for periodontal tissues in vitro [17], while positive therapeutic effects were seen in two clinical studies in the field of periodontal therapy [18, 19]. Furthermore, the intake of vitamins C and D seem to play an important role in gingival and periodontal inflammation. Several studies showed the positive impact of vitamin D on periodontal tissues both in clinical and in-vitro studies [11, 20, 21]. Vitamin C has been described as an important vitamin for periodontal health, both in clinical and in-vitro studies for some time. The absence of vitamin C causes scurvy, which is accompanied by massive periodontal bone loss [22–25]. Last but not least, the role of dietary antioxidants seems to be important for several processes regarding an adequate systemic reaction to oxidative stress. Both clinical and in-vitro studies showed positive effects on periodontal tissues [11, 12, 26–28]. Despite these very promising findings, there is a substantial lack of dietary-interventional studies in controlled randomized settings [11]. Thus, the aim of this pilot study was to evaluate an oral health optimized diet low in carbohydrates, and rich in Omega 3-fatty acids, vitamins C and D, antioxidants and rich in fiber in a controlled, randomized study.

**Methods**  
**Ethics and trial registration** Prior to patient recruitment the study was approved by the University of Freiburg Ethics committee (Reference number 338/14) and registered in an international clinical trial register (German Clinical Trials Register; DRKS00006301; <https://www.germanctr.de/>).

**Inclusion criteria**

- age  $\geq$  18 years – patients with gingivitis ( $GI > 0,5$ ) – a diet based primarily on carbohydrates [29]

**Exclusion criteria**

- smoking – infectious or life-threatening diseases – intake of antibiotics within 3 months before the start of or during the study period. – drugs influencing gingival inflammation or

bleeding (e.g. anticoagulants, cortisone) – carbohydrate- or insulin-related diseases (e.g. diabetes) – pregnancy or breastfeeding

**Patient recruitment** Patients were recruited in the Department of Operative Dentistry and Periodontology, Medical Center - University of Freiburg, Germany. Freiburg is located in the southwest of Germany with approximately 220.000 inhabitants. Patients were informed about the study and in case of gingival inflammation assessed using the gingival index by Löe & Silness [30] asked for participation. At this stage no further periodontal assessment was performed. Exclusion criteria were checked (by CT) including the prerequisite of a diet mainly based on carbohydrates by means of a verbal dietary anamnesis. After receiving written consent, participants were randomly assigned to the experimental group or the control group following a web-generated randomization list. **Dietary recommendations** Dietary recommendations were based on the current literature with regard to diet and general inflammation [9, 31], and gingival / periodontal inflammation [7, 11]. The dietary pattern was finally checked by a specialist in internal medicine, nutrition and diabetology (DK). Dietary pattern in the experimental group included the following elements:

- Reduction of the intake of carbohydrates as far as possible to a level <130 g/d, which can be considered as a low-carb diet [29]. This included a restriction in the amount of fructose, disaccharides, sweetened beverages and meals, flour containing foods, rice and potatoes as far as possible. There were no restrictions regarding fruits and vegetables (polysaccharides) as long as the total amount of carbohydrates was considered.
- Daily intake of Omega-3 fatty acids (such as fish oil capsules, a portion of sea fish, two spoons of flaxseed oil etc.), a restriction in the amount of trans-fatty acids as far as possible (such as fried meals, crisps, donuts, croissants etc.) and a reduction in Omega-6 fatty acids as far as possible (such as safflower oil, grape seed oil, sunflower oil, margarine, sesame oil, corn oil etc.).
- Daily intake of a source of vitamin C (like two kiwis, one orange, one bell pepper etc.)
- Daily intake of a source of vitamin D (15 min unprotected in the sun, supplementation with 500 international units (12.5 µg), 300 g Avocado, etc.).
- Daily intake of antioxidants (such as a handful of berries, cup of green tea, coffee etc.)
- Daily intake of fiber (vegetables and fruits).

Dietary recommendations were delivered verbally (30 min) and by handing out an information brochure containing an additional list of restricted and recommended foods and meals. After one week, participants were asked about their experiences and possible problems. When more information was needed, participants had the chance to contact two of the authors at any time during the study (JPW, CT). All participants had to fill out a daily food diary (Additional file 1) throughout the study duration, and to return the diaries at the current appointment.

**Clinical measurements and procedures** Clinical measurements were performed by a dentist blinded to the kind and group of intervention (KB) and included assessment of a gingival index (GI by Löe & Silness [30], plaque index (PI by Silness & Löe [32], a full-mouth periodontal examination including pocket probing depths (PD), bleeding on probing (BOP), and recessions at six sites of the teeth respectively. The periodontal status was assessed using a pressure-sensitive probe (DB764R, Aesculap AG, Tuttlingen, Germany). After assessing the full-mouth periodontal status the first quadrant was examined twice in order to calculate the intrarater variance. The data were saved digitally via specialized

periodontal software (Parostatus.de, Parostatus.de GmbH, Berlin, Germany), which was also used to calculate the total periodontal inflamed surface area (PISA) according to Nesse et al. [33]. For both groups the baseline was assessed in two appointments after one and two weeks. At the start of the first week all participants were instructed to stop all interdental hygiene procedures for the next eight weeks. The first baseline measurement included oral hygiene indices (GI, PI). At the second appointment oral hygiene indices were assessed once again and the periodontal status (PD, BOP, recessions) was evaluated. Participants were encouraged not to change their physical activity and oral hygiene behavior during the entire study, and to stop any interdental cleaning. In case of detected periodontal lesions participants were informed about their disease and scheduled for periodontal treatment after the study. After the final baseline measurement, patients of the experimental group had an appointment for the dietary instructions with one of the authors (JPW, CT). The following two weeks were seen as a transition time for the patients in order to identify possible problems in changing their dietary behavior to the new diet. After these two transition weeks, patients of the experimental group were encouraged to follow the oral health optimized diet patterns for four weeks to the best of their ability. This observation period was based on the study by Baumgartner et al. [6]. In this period, GI and PI were assessed weekly. At the end of the four weeks total periodontal status was again assessed. As a reward for participating in the study, the patients were given an electric toothbrush with a value of about 70 Euro. Patients in the control group were evaluated using the same schedule, but were encouraged to continue their dietary habits. All participants were measured in height and weight in order to calculate the Body Mass Index (BMI). Furthermore, patients were encouraged not to talk to the rating dentist (KB) about study related content. The food diaries were analyzed with regard to the degree of the participants' compliance in fulfilling the recommended diet.

Statistical procedures Sample size calculation was based on the Baumgartner et al. [6] study with a reduction in BOP from 34.8 ( $\pm 24.3$ ) to 12.6 ( $\pm 10$ ). Accordingly, with a power = 80 % and alpha = 5 %,  $n=12$  participants would be needed. Due to the assumption of correlated participants (population of family members) in the Baumgartner study,  $n=10$  participants were aimed for current sample size in an independent population. The control group of  $n=5$  participants was not determined by sample size calculation. The study was considered as a pilot study. Mean BOP was considered as the primary outcome variable, GI, PI, PISA as secondary outcome variables. Randomization was performed by one of the authors (JPW) using a web-based randomizer ([www.random.org](http://www.random.org), Randomness and Integrity Services Ltd., Dublin, Ireland) with a presetting of five controls and ten experimentals. The descriptive analysis included median, mean, standard deviation and minimum and maximum. For testing the differences between both groups, a mixed linear regression analysis was performed. Multiple testing was corrected using the Scheffe method. Intra-rater variance was analyzed by calculating the intraclass correlation coefficient (ICC) for the rating dentist (KB). All analyses were performed with a statistical software (STATA 13.1, StataCorp, Texas, USA).

Results In total, 16 patients with gingivitis were recruited. In the experimental group, one participant dropped out of the study due to a lack of time for study participation. The experimental group consisted of 10 participants with 6 women and 4 men. The mean age in this group was  $34.4 \pm 14.1$  years, ranging from 23 to 70 years. The mean BMI was  $24.53 \pm 2.53$  kg/m<sup>2</sup>. The control group consisted of 5 participants with 3 women and 2 men.

The mean age in this group was  $34.0 \pm 16.5$  years, ranging from 24 to 63 years. The mean BMI was  $21.98 \pm 3.24$  kg/m<sup>2</sup>. Clinical data regarding PI, GI, PD, CAL, BOP and PISA are presented in Table 1. A flow chart of the procedures is presented in Fig. 1. In the experimental group, one patient suffered from mild periodontitis and one patient from moderate periodontitis [34]. In the control group, two patients suffered from mild periodontitis. Regression analysis adjusted for age, gender and BMI revealed no significant differences between the groups regarding PI ( $p=0.084$ ), but significant differences regarding GI ( $p<0.001$ ), BOP ( $p=0.012$ ), and PISA ( $p<0.001$ ). Table 2 presents the degree of compliance regarding dietary recommendations. A regression analysis regarding the influence of the degree of compliance on the clinical parameters was performed for PI, GI, BOP, and PISA (Table 3). According to the regression analysis PI was significantly negatively associated with Omega-3 fatty acids and positively associated with fiber intake. GI was significantly negatively associated with Omega-3 fatty acid consumption and carbohydrate reduction. BOP and PISA were both significantly negatively associated with a reduction in carbohydrates. Intraclass Correlation was 0.91, showing a high level of reproducibility. The computed power of the study was 100 %.

**Discussion** The results of this pilot study showed that an oral health optimized diet can significantly reduce gingival and periodontal inflammation in a clinically important range without any changes in oral hygiene performance. The results of this study are in accordance with those of Baumgartner et al. [6], questioning the positive association of plaque and gingival inflammation in a changed dietary environment. Although the overall effects of this diet were impressive, it is hard to determine in retrospect which dietary element had the most impact on clinical parameters, even though the regression analysis showed significant associations of clinical factors primarily to carbohydrate-reduction and Omega-3 fatty acid intake. The control group showed a trend of increasing plaque values although this was not significant. This result was expectedly due to the absence of interdental hygiene during the study in individuals who prior to this performed interdental hygiene. But also irrespectively to the control group, the experimental group experienced a significant decrease in inflammatory parameters with regard to the baseline values, which is comparable with the Baumgartner et al. study. In total, the results support the assumptions that a modern, Western lifestyle, including lots of refined carbohydrates and a high Omega-6 to Omega-3 fatty acid ratio promotes inflammatory processes [8]. As long as no specific dietary component can be made out for this decrease in periodontal inflammation, results have to probably be considered to be a mixture of inhibition and increased resolution of the inflammatory processes. Regarding the intake of high-glycemic carbohydrates which promotes higher blood glucose levels and insulinemia, the results of this study seem to be in line with

Table 1 Clinical data of both groups for all weeks

Factor	Group	Week 1	Week 2	Week 5	Week 6	Week 7	Week 8
PI	Experimental	0.77 (0.52)	0.88 (0.48)	0.89 (0.51)	0.85 (0.46)	0.80 (0.52)	0.84 (0.47)
	Control	0.75 (0.63)	0.81 (0.46)	0.94 (0.51)	0.84 (0.53)	0.88 (0.56)	0.97 (0.70)
GI	Experimental	1.10 (0.51)	1.20 (0.30)	0.80 (0.42)	0.62 (0.37)	0.54 (0.39)	0.54 (0.30)
	Control	1.01 (0.14)	1.04 (0.17)	1.30 (0.17)	1.38 (0.15)	1.30 (0.19)	1.22 (0.17)
PD	Experimental	- 2.19 (0.34)	- - - 2.11 (0.35)	Control - 2.31 (0.43)	- - - 2.52 (0.40)		
	Control	- 2.31 (0.52)	- - - 2.22 (0.47)	Control - 2.53 (0.90)	- - - 2.76 (0.88)		
BOP (%)	Experimental	- 53.57 (18.65)	- - - 24.17 (11.57)	Control - 46.46 (15.61)	- - - 64.06 (11.27)		
	Control	- 638.88 (305.41)	- - - 284.83 (174.14)	Control - 662.24 (420.05)			

--- 963.24 (373.78) PI Clinical results regarding plaque index, GI gingival index, PD pocket depth, CAL clinical attachment level, BOP bleeding on probing, and PISA periodontal inflamed surface areas with mean values with the standard deviations in parentheses the known relationship of periodontal disease and diabetes [35] and the recently investigated periodontitis-associated genes ANRIL, VAMP3, and GLT6D1 which in some way seem to be related to glucose metabolism or glycosylation [36–38]. The association of carbohydrate consumption and gingivitis has been investigated in some earlier studies, also with impressive effects [7]. Looking into the literature there are several possible explanations for the inflammation triggering effect of carbohydrates. First of all, high glycemic index carbohydrates seem to

Table 2 Degree of compliance to dietary recommendations Factor Group Week 1 Week 2 Week 5 Week 6 Week 7 Week 8 Vitamin C Experimental 0.46 (0.29) 0.49 (0.25) 0.70 (0.37) 0.80 (0.20) 0.76 (0.30) 0.79 (0.28) Control 0.56 (0.20) 0.56 (0.25) 0.56 (0.32) 0.50 (0.34) 0.40 (0.31) 0.46 (0.22) Omega-3 fatty acids Experimental 0.04 (0.10) 0.07 (0.11) 0.82 (0.33) 0.82 (0.36) 0.87 (0.30) 0.83 (0.33) Control 0.24 (0.43) 0.24 (0.43) 0.24 (0.43) 0.24 (0.43) 0.22 (0.44) 0.22 (0.44) Fibers Experimental 0.40 (0.29) 0.38 (0.27) 0.90 (0.17) 0.88 (0.16) 0.94 (0.14) 0.93 (0.15) Control 0.50 (0.19) 0.64 (0.30) 0.44 (0.27) 0.48 (0.39) 0.48 (0.38) 0.48 (0.38) Vitamin D Experimental 0.13 (0.31) 0.11 (0.31) 0.41 (0.45) 0.42 (0.48) 0.43 (0.47) 0.47 (0.46) Control 0.24 (0.43) 0.22 (0.44) 0.22 (0.44) 0.22 (0.44) 0.32 (0.46) 0.22 (0.44) Antioxidants Experimental 0.70 (0.42) 0.76 (0.41) 0.87 (0.29) 0.77 (0.42) 0.93 (0.22) 0.89 (0.31) Control 0.60 (0.55) 0.48 (0.38) 0.56 (0.45) 0.62 (0.41) 0.58 (0.36) 0.54 (0.39) Carbohydrate reduction Experimental 0.18 (0.19) 0.10 (0.09) 0.88 (0.21) 0.92 (0.11) 0.96 (0.07) 0.98 (0.04) Control 0.34 (0.11) 0.34 (0.09) 0.30 (0.20) 0.32 (0.16) 0.32 (0.27) 0.32 (0.13) Degree of compliance for dietary recommendations after analyzing the food diaries. Values are given in mean with the standard deviations in parentheses. [0=no compliance, 1=consumption 100 % as recommended]

Table 3 Regression analysis regarding clinical factors and degree of compliance Clinical factor Dietary factor Coefficient Standard deviation p-value 95 % confidence interval PI Vitamin C 0.12 0.09 0.181 [-0.56; 0.30] Omega-3 FA -0.26 0.11 0.022 [-0.49; -0.04] Fibers 0.33 0.15 0.021 [0.05; 0.63] Vitamin D -0.10 0.83 0.815 [-0.18; 0.14] Antioxidants -0.05 0.10 0.589 [-0.25; 0.14] Carbohydrate reduction -0.11 0.13 0.375 [-0.38; 0.14] GI Vitamin C -0.08 0.10 0.452 [-0.28; 0.12] Omega-3 FA -0.42 0.12 0.001 [-0.69; -0.18] Fibers 0.01 0.16 0.952 [-0.31; 0.33] Vitamin D 0.15 0.09 0.089 [-0.02; 0.32] Antioxidants -0.20 0.11 0.272 [-0.33; 0.09] Carbohydrate reduction -0.59 0.15 0.001 [-0.88; -0.29] BOP Vitamin C -0.09 0.11 0.394 [-0.30; 0.12] Omega-3 FA -0.04 0.09 0.68 [-0.21; 0.14] Fibers 0.22 0.14 0.11 [-0.05; 0.49] Vitamin D 0.01 0.07 0.95 [-0.14; 0.15] Antioxidants -0.16 0.09 0.06 [-0.33; 0.01] Carbohydrate reduction -0.47 0.14 0.01 [-0.75; -0.29] PISA Vitamin C -59.03 147.25 0.689 [-347.64; 229.58] Omega-3 FA -229.65 136.18 0.092 [-496.43; 37.14] Fibers 239.73 200.92 0.233 [-154.07; 633.53] Vitamin D 113.11 113.65 0.320 [-109.64; 335.86] Antioxidants -87.78 125.79 0.485 [-334.33; 158.76] Carbohydrate reduction -581.59 197.88 0.003 [-969.42; -193.76]

directly promote inflammatory processes via NFκB activation and oxidative stress [39, 40], and have been linked to higher C-reactive protein levels [41]. Secondly, high glycemic index

carbohydrates may promote weight gain [42], with its associated inflammatory effects, based on an increase in adipokine secretion [43]. Looking at mean carbohydrate consumption in Germany, with more than 230 g per day or over 45 % of the total calorie intake [44], there may be associations with chronic inflammatory diseases in susceptible patients. Furthermore, the results are in line with Sidi & Ashley [45] who found a significant higher bleeding on probing in individuals on a high sugar diet compared to individuals on a low sugar diet, with no intergroup differences regarding the amount of plaque. Regarding the effect of a decrease in the Omega-6 to Omega-3 fatty acid ratio, our results support the theory of resolomics as stated by Serhan et al. [9], and the related periodontal studies [18, 19, 46]. Summarized, Serhan and colleagues discovered that the resolution of the inflammatory response is an active process based on metabolites of Omega-3 fatty acids, so called “specialized pro-resolving mediators” (SPM), rather than a passive event based on the elimination of the pro-inflammatory cytokines. Looking at the intake of vitamins C and D and antioxidants, the results also support studies which have shown positive outcomes in this field. For a deeper discussion a reference should be made to Van der Velden et al. [11], where theoretical and clinical studies are described in detail. The results raise several questions regarding the importance of dental plaque for the development of gingivitis/ periodontitis and its impact on therapy. If dental examination reveals signs of an inflammation of the gingiva or the periodontium, representing a strongly host-mediated condition, a primary check of host-factors such as nutrition should be considered. The periodontist has the special opportunity – in contrast to that of other medical professions – to fairly easily get an impression about systemic inflammation, which could be a real contribution from periodontology in the prevention of other diseases related to chronic inflammation. This idea has been stated from by other authors as well [7, 47]. The primary limitations of this study can be seen in its small sample size, which is not a representative population, as well as with the rather uncontrolled intake of dietary components. However, to the best of the authors’ knowledge the study results are the currently only evidence beyond the Baumgartner et al. study in a randomized, controlled, interventional setting. Regarding the rather broad dietary recommendations, in the authors’ opinion it is very important to deliver a dietary protocol which is applicable to patients in daily practice. In other words, advising patients to implement this dietary pattern will show effects irrespective of whether they focus more on high glycemic index carbohydrate reduction, Omega-3 fatty acids, vitamin D, vitamin C, and/or antioxidants. Future studies should also include serum measurements of cytokines, cholesterol, HbA1c, vitamin D, etc. and also examine the oral microbiome. According to the exclusion criteria the study results are limited to patients with a mainly carbohydrate based diet. With regard to European epidemiological data, it can be assumed that this factor also applies to most individuals in the European population [48]. Furthermore, the assessment of dietary patterns using food diaries is not absolutely accurate. Thus, the presented data based on the food diaries can be seen as an indicator of compliance but not as a precise reflection of dietary patterns including quantitative values.

**Conclusion** Within the limitations, the presented dietary pattern including a diet low in carbohydrates, but rich in Omega-3 fatty acids, vitamins C and D, antioxidants and fiber significantly reduced periodontal inflammation in humans.

Additional files

Additional file 1: “S1 food diary.doc” contains the used food diary. (DOC 155 kb) Additional file 2: “Dataset.xlsx” contains the total anonymized dataset including group affiliation, gender, age, BMI, PI, GI, BOP, PISA, PD, CAL, and compliance values regarding the recommended nutrition. (XLSX 17 kb)

Abbreviations GI, gingival index; PI, plaque index; PD, pocket probing depths; BOP, bleeding on probing; PISA, periodontal inflamed surface area; BMI, body mass index; ICC, intraclass correlation coefficient.

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Availability of data and materials The dataset supporting the conclusions of this article is included within the article Additional file 2.

Authors' contributions JPW, CT, KB, DK, EH, AA, PRK conceived of the study, and participated in its design and helped to draft the manuscript. KB carried out the clinical measurements. KV performed statistical analysis. All authors participated in the writing process, read and approved the final manuscript.

Competing interests The authors declare that they have no competing interests.

Consent for publication Not applicable.

Ethics approval and consent to participate Prior to patient recruitment the study was approved by the University of Freiburg Ethics committee (Reference number 338/14) and registered in an international clinical trial register (German Clinical Trials Register; DRKS00006301; <https://www.germanctr.de/>). All participants gave their written consent.

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8. Which statement is false. For a product to be certified organic, it's required to meet these requirements:

- a. Organic crops cannot be grown with synthetic fertilizers, synthetic pesticides or sewage sludge.
- b. Organic crops cannot be genetically engineered or irradiated.
- c. Animals must eat organically grown feed at least 70% of the time.
- d. Animals have access to the outdoors, and ruminants (hoofed animals, including cows) must have access to pasture.

What is organic?

Organic is a labeling term that indicates that the food or other agricultural product has been produced and processed using approved methods. These methods integrate cultural, biological, and mechanical practices that foster cycling of resources, promote ecological balance, and conserve biodiversity. Synthetic fertilizers, sewage sludge, irradiation, and genetic engineering may not be used.

Who is affected by the USDA organic regulations?

All products that are sold, labeled, or represented as organic must meet all requirements in the USDA organic regulations. Products must be certified organic by a USDA-accredited certifying agent. The USDA organic regulations ensure that organically labeled products meet consistent national standards.

Exceptions. Operations whose gross agricultural income from organic sales is less than \$5,000 do not need to be certified in order to sell, label, or represent their products as organic. These operations also do not need to prepare an organic systems plan. However, they must still comply with all other USDA organic regulations. Exempt operations may use the word "organic," but may not use the USDA organic seal on their products. Retail food establishments that sell organically produced agricultural products do not need to be certified.

What are the USDA organic requirements?

The organic standards describe the specific requirements that must be verified by a USDA accredited certifying agent before products can be labeled organic. An overview of some of the crops, livestock, and handling standards are provided below. Please note that all organic operations must comply with all requirements in the USDA organic regulations.

Crop Standards

The organic crop production standards require that:

- Land must have had no prohibited substances applied to it for at least 3 years before the harvest of an organic crop.
- Soil fertility and crop nutrients will be managed through tillage and cultivation practices, crop rotations, and cover crops, supplemented with animal and crop waste materials and allowed synthetic materials.
- Crop pests, weeds, and diseases will be controlled primarily through management practices including physical, mechanical, and biological controls. When these practices are not sufficient, a biological, botanical, or synthetic substance approved for use on the National List may be used.
- Operations must use organic seeds and other planting stock when available.

● The use of genetic engineering, ionizing radiation and sewage sludge is prohibited.  
National Organic Program | U.S. Department of Agriculture | [www.ams.usda.gov/nop](http://www.ams.usda.gov/nop) | 202-720-3252

#### Organic Production and Handling Standards

#### Organic Production and Handling Standards (continued)

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#### Livestock and Poultry Standards

Livestock and poultry standards apply to animals used for meat, milk, eggs, and other animal products sold, labeled, or represented as organic. Some requirements include:

- Animals for slaughter must be raised under organic management from the last third of gestation, or no later than the second day of life for poultry.
- Producers must feed livestock agricultural feed products that are 100 percent organic, but they may also provide allowed vitamin and mineral supplements.
- Dairy animals must be managed organically for at least 12 months in order for milk or dairy products to be sold, labeled or represented as organic.
- Preventive management practices must be used to keep animals healthy. Producers may not withhold treatment from sick or injured animals. However, animals treated with a prohibited substance may not be sold as organic.
- Ruminants must be out on pasture for the entire grazing season, but for not less than 120 days. These animals must also receive at least 30 percent of their feed, or dry matter intake (DMI), from pasture.
- All organic livestock and poultry are required to have access to the outdoors year-round. Animals may only be temporarily confined due to documented environmental or health considerations.
- Organically raised animals must not be given hormones to promote growth or antibiotics for any reason.

#### Handling Standards

The handling standards require:

- All non-agricultural ingredients, whether synthetic or non-synthetic, must be allowed according to the National List of Allowed and Prohibited Substances.
- In a multi-ingredient product labeled as “organic,” all agricultural ingredients must be organically produced, unless the ingredient(s) is not commercially available in organic form and listed on Section 205.606.
- Handlers must prevent the commingling of organic with non-organic products and protect organic products from contact with prohibited substances.

#### Labeling Multi-Ingredient Products

- Products sold, labeled, or represented as organic must have at least 95 percent certified organic content.
- Products sold, labeled, or represented as “made with” organic must have at least 70 percent certified organic content. The USDA organic seal may not be used on these products.
- Products containing less than 70 percent organic content may identify specific ingredients as organic in the ingredients list.

**9. Which statement is false:**

- a. Human studies have shown that a diet that eliminates free-sugars and processed grains and includes healthy foods can reduce bleeding-on-probing and reduce overgrowth of pathogenic in dental plaque.
- b. Human studies have shown that dental plaque has pathogenic bacteria that must be removed completely.
- c. Human studies have shown that anaerobic bacteria on the surface of the tongue are necessary for the natural production of nitric oxide and reduction of blood pressure.
- d. Daily use of an antimicrobial mouthwash could disturb the healthy balance of bacteria in the mouth and lead to damage in the mouth.

**See 1<sup>st</sup> Article of Question 7**

Oral Microbiome and Nitric Oxide: the Missing Link in the Management of Blood Pressure

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Abstract Having high blood pressure puts you at risk for heart disease and stroke, which are leading causes of death in the USA and worldwide. One out of every three Americans has hypertension, and it is estimated that despite aggressive treatment with medications, only about half of those medicated have managed blood pressure. Recent discoveries of the oral microbiome that reduces inorganic nitrate to nitrite and nitric oxide provide a new therapeutic target for the management of hypertension. The presence or absence of select and specific bacteria may determine steady-state blood pressure levels. Eradication of oral bacteria through antiseptic mouthwash or overuse of antibiotics causes blood pressure to increase. Allowing recolonization of nitrate- and nitrite-reducing bacteria can normalize blood pressure. This review will provide evidence of the link between oral microbiota and the production of nitric oxide and regulation of systemic blood pressure. Management of systemic hypertension through maintenance of the oral microbiome is a completely new paradigm in cardiovascular medicine.

Keywords Gut microbiome . Blood pressure . Hypertension . Nitric oxide . Diet . Nitrate . Nitrite  
Hypertension

Human blood pressure regulation is complex, and despite decades of research into the variables affecting resting blood pressure, there is still a significant knowledge gap. Hypertension is highly prevalent in the adult population in the USA, especially among persons older than 60 years of age and affects approximately one billion adults worldwide [1, 2]. In the USA, about 77.9 million (one out of every three) adults have high blood pressure. Among persons 50 years of age or older, isolated systolic hypertension is the most common form of hypertension [4, 5], and systolic blood pressure becomes more important than diastolic blood pressure as an independent risk predictor for most all cardiovascular-related disease including end-stage renal disease (ESRD) [6–9]. In some age groups, the risk of cardiovascular disease doubles for each increment of 20/10 mmHg of blood pressure, starting as low as 115/75 mmHg. In addition to coronary heart diseases and stroke, complications of raised blood pressure include heart failure, peripheral vascular disease, renal impairment, retinal hemorrhage, and visual impairment. Worldwide, raised blood pressure is estimated to cause 7.5 million deaths, about 12.8% of the total of all deaths. This accounts for 57 million disability adjusted life years (DALYS) or 3.7% of total DALYS. Globally, the overall prevalence of raised blood pressure in adults aged 25 and over was around 40% in 2008. The proportion of the world's population with high blood pressure, or uncontrolled hypertension, fell modestly between 1980 and 2008. However, because of population growth and aging, the number of people with uncontrolled hypertension rose from 600 million in 1980 to nearly one billion in 2008 [10]. Despite major advances in understanding the

pathophysiology of hypertension and availability of antihypertensive drugs, suboptimal blood pressure control is still the most

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important risk factor for cardiovascular mortality. According to the AHA 2013 Statistics Fact Sheet, although 75% of people that know they have hypertension and are under current treatment, only about 52% of those have it controlled. The Systolic Blood Pressure Intervention Trial (SPRINT) showed that among adults with hypertension but without diabetes, lowering systolic blood pressure to a target goal of less than 120 mmHg, as compared with the standard goal of less than 140 mmHg, resulted in significantly lower rates of fatal and nonfatal cardiovascular events and death from any cause [11]. Because blood pressure remains elevated in ~50% of all treated hypertensive patients [12, 13], and more effective blood pressure management can save lives, novel, efficacious, and cost-effective therapeutic strategies are urgently required for the treatment of hypertension.

Nitric Oxide

The discovery of endothelium-derived relaxing factor (EDRF) in 1980 [14••] that was later identified as nitric oxide (NO) [15•, 16•] revolutionized vascular biology and identified new strategies for controlling blood pressure. NO is produced endogenously from the five-electron oxidation of the guanidino nitrogen of L-arginine by the enzyme isoform endothelial nitric oxide synthase (eNOS) [17]. NO produced or generated in the vasculature then diffuses into the underlying smooth muscle causing these muscles to relax. This results in vasodilation, causing a reduction in systemic blood pressure and an increase in blood flow and oxygen delivery to specific vascular beds. In healthy subjects, activation of eNOS causes vasodilation in both muscular conduit vessels and resistance arterioles. In contrast, in subjects with atherosclerosis or endothelial dysfunction, similar stimulation yields attenuated vasodilation in peripheral vessels and causes paradoxical vasoconstriction in coronary arteries, thus indicating a decrease in the production and/or bioavailability of NO [18, 19]. Emerging evidence shows that endothelial dysfunction and subsequent NO deficiency are critically associated with the development of hypertension and other forms of cardiovascular disease [20]. Hypertension accelerates atherosclerosis. Interestingly, endothelial dysfunction can be demonstrated in patients with risk factors for atherosclerosis in the absence of atherosclerosis itself [21, 22]. Experimental and clinical studies provide evidence that defective endothelial NO function is not only associated with all major cardiovascular risk factors, such as hyperlipidemia, diabetes, hypertension, smoking, and the severity of established atherosclerosis but also has a profound predictive value for future atherosclerotic disease progression [23]. These results indicate that essential hypertension is characterized by an age-related reduction of nitric oxide production and bioavailability.

Human Oral Microbiome—Bacterial Nitrate Reduction

The human microbiome is composed of many different bacterial species, which outnumber our human cells ten to one and provide functions that are essential for our survival. The microbiota of the lower intestinal tract is widely recognized as playing a symbiotic role in maintaining a healthy host physiology [24] by participating in nutrient acquisition and bile acid recycling, among other activities. In contrast, although the role of oral microbiota in disease is well studied, specific contributions to host health are not well defined. A human nitrogen cycle has been identified. This pathway, termed the enterosalivary nitrate-nitrite-nitric oxide pathway, can positively affect nitric oxide homeostasis and represents a potential symbiotic relationship between oral bacteria and their human hosts [25, 26]. It is now recognized

that the oral commensal bacteria provide an important metabolic function in human physiology by contributing a nitric oxide synthase (NOS)-independent source of NO. This process is analogous to the environmental nitrogen cycle, whereby soil bacteria convert atmospheric nitrogen oxides to usable forms for plant growth. Human nitrate reduction requires the presence of nitrate-reducing bacteria as mammalian cells cannot effectively reduce this anion. Inorganic nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) are known as food additives to cured meats and naturally occurring in green leafy vegetables [27, 28] but also as oxidative end products of endogenous NO production [29]. However, from research performed over the past decade, it is now apparent that nitrate and nitrite are physiologically recycled in blood and tissue to form NO and other bioactive nitrogen oxides [30, 31–33] and now considered essential nutrients [34, 35]. As a result, they should now be viewed as storage pools for NO-like bioactivity to be acted upon when enzymatic NO production from NOS is insufficient, such as during anaerobic conditions or uncoupling of NOS. The bioactivation of nitrate from dietary (mainly green leafy vegetables) or endogenous sources (oxidation of NO) requires its initial reduction to nitrite, and because mammals lack specific and effective nitrate reductase enzymes, this conversion is mainly carried out by commensal bacteria [36]. Dietary nitrate is rapidly absorbed in the upper gastrointestinal tract. In the blood, it mixes with the nitrate formed from the oxidation of endogenous NO produced from the NOS enzymes. After a meal rich in nitrate, the nitrate levels in plasma increase greatly and remain high for a prolonged period of time (the plasma half-life of nitrate is 5–6 h). The nitrite levels in plasma also increase after nitrate ingestion after approximately 90 min [37] provided it can be reduced by oral nitrate-reducing bacteria. Although much of the nitrate is eventually excreted in the urine, up to 25% is actively taken up by the salivary glands and is concentrated up to 20-fold in saliva [37, 38]. In the mouth, commensal facultative anaerobic bacteria reduce salivary nitrate to nitrite during anaerobic respiration by the action of nitrate reductases [26, 36]. After a dietary nitrate load, the salivary nitrate and nitrite levels can approach 10 mM and 1–2 mM, respectively [37]. When saliva enters the acidic stomach (1–1.5 L per day), much of the nitrite is rapidly protonated to form nitrous acid (HNO<sub>2</sub>; pK<sub>a</sub> 3.3), which decomposes further to form NO and other nitrogen oxides [32, 33]. A simplified human nitrogen cycle is illustrated in Fig. 1. More recent studies indicate that nitrite does not have to be protonated to be absorbed and is about 98% bioavailable when swallowed in an aqueous solution [39]. Salivary nitrate is metabolized to nitrite via a two-electron reduction, a reaction that mammalian cells are unable to perform, during anaerobic respiration by nitrate reductases produced by facultative and obligate anaerobic commensal oral bacteria [40, 41]. As illustrated in Fig. 2, identification of specific partial denitrifying bacteria in the oral cavity can provide optimal conditions for nitrate reduction with nitrite accumulation in the saliva. Although a few nitrate-reducing bacteria have been identified in the oral cavity [42], we have analyzed nitrate reduction by bacterial communities present in tongue scrapings from healthy human volunteers during 4 days of *in vitro* growth and performed a parallel metagenomic analysis of these samples to identify specific bacteria associated with nitrate reduction. Through 16S rRNA gene pyrosequencing and whole genome shotgun (WGS) sequencing and analysis, we identified specific taxa that likely contribute to nitrate reduction. Biochemical characterization of nitrate and nitrite reduction by four candidate species indicates that complex community interactions contribute to nitrate reduction [43]. The bacterial communities had varying potential for nitrate reduction, and our study identified 14 candidate species that were present in communities with the best nitrate reduction activity. The 14 species present at an abundance of at least 0.1% in the best nitrate-reducing sample and at the highest abundance in this sample compared to the intermediate and worst reducing sample that belonged to the genera of interest and were identified through 16S rRNA gene pyrosequencing and analysis were *Granulicatella adiacens*, *Haemophilus parainfluenzae*,

*Actinomyces odontolyticus*, *Actinomyces viscosus*, *Actinomyces oris*, *Neisseria flavescens*, *Neisseria mucosa*, *Neisseria sicca*, *Neisseria subflava*, *Prevotella melaninogenica*, *Prevotella salivae*, *Veillonella dispar*, *Veillonella parvula*, and *Veillonella atypica*. Additionally, *Fusobacterium nucleatum* and *Brevibacillus brevis* were designated as species of interest even though they were not at a relative abundance of at least 0.1% in the WGS best nitrate-reducing sample [43]. Previously, the Doel et al., 2005, study isolated and identified five genera of oral nitrate-reducing bacterial taxa on the tongues of healthy individuals: *Veillonella*, *Actinomyces*, *Rothia*, *Staphylococcus*, and *Propionibacterium* [42]. In our investigation, *Veillonella* species were the most abundant group of nitrate reducers isolated from the tongue, followed by *Actinomyces* spp. *Veillonella* was the most abundant nitrate-reducing genus detected in the original tongue scrapings, although *Prevotella*, *Neisseria*, and *Haemophilus* were all found at a higher abundance than *Actinomyces*, highlighting the higher resolution of our study. This difference in resolution is likely due to our use of a sequencing-based approach, which allowed us to survey the native bacterial environment on the dorsal surface of the

NO<sub>3</sub>

NO<sub>2</sub>

NO

oxidation reduction

BACTERIA 2 e<sup>-</sup> reduction

Acid Deoxy-hemoproteins

DIET

Oxygen, ceruloplasmin

Oxyheme proteins

L-arginine

NOS

BACTERIA 1 e<sup>-</sup> reduction

Fig. 1 The human nitrogen cycle whereby nitrate is serially reduced to nitrite and NO providing the host with a source of bioactive NO

Fig. 2 The biological nitrogen cycle. Nitrate is reduced all the way down to elemental nitrogen by a series of enzymatic steps from nitrate reductase (NR), nitrite reductase [3], NO reductase (NOR), and nitrous oxide reductase (N<sub>2</sub>OR). Identifying bacteria or communities that only reduce nitrate and/or nitrite will allow for nitrite accumulation and more efficient NO

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tongue without depending on the growth requirement necessary for classic culture-based techniques. Metagenomic data are important and informative in that one can determine what a bacterial community is capable of doing, yet it is limited in the sense that it cannot inform what the community is actually doing, which can vary under different circumstances. Thus, while each community had the same capacity for nitrate reduction, the true activity clearly differed between these communities. Furthermore, as not all healthy donors had nitrate-reducing bacteria in their oral cavity, there may be a significant number of individuals who may not have a functioning nitrate-nitrite-NO enterosalivary pathway to support systemic health. The presence or absence of these select bacteria may be a new determinant of nitrite and NO bioavailability in humans and, thus, a new consideration for cardiovascular disease risk. Further studies on multi-species biofilms integrating biochemical, metagenomic, and metatranscriptomic data will answer these important questions and provide more information regarding the community dynamics that contribute to oral nitrate reduction that results in nitrite accumulation.

Nitrite-Based Signaling

The production of nitrite from nitrate-reducing bacteria may have profound implications on the health of the human host. Numerous studies have shown that nitrite produced from bacterial nitrate

reduction is an important storage pool for NO in blood and tissues when NOS-mediated NO production is insufficient [44•, 45–48]. Nitrite is also an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO flux/formation [49]. Nitrite is now recognized as a cell signaling molecule that can act as a storage form of NO as well as a NO-independent signal [30•, 50]. Nitrite is in steady-state equilibrium with S-nitrosothiols [30•, 51] and has been shown to activate soluble guanylyl cyclase (sGC) and increase cGMP levels in tissues [30•] and lead to vasodilation [52, 53]. Therefore, it is an ideal candidate for restoring both cGMP-dependent and cGMP-independent NO signaling. In addition to the oxidation of NO, nitrite is also derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract [54, 55], as well as from dietary sources, such as meat, vegetables, and drinking water. Since nitrate reduction by microbial communities generates nitrite, it is of great importance to be able to recognize specific bacteria in and on the body that are capable of generating nitrite from nitrate. Once nitrite is formed, it can be utilized as a substrate for NO production. Although largely inefficient, there exists a number of nitrite-reducing systems in mammals. Nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system [56, 57], protonation [31], deoxyhemoglobin [58], and xanthine oxidase [59, 60]. Nitrite can also transiently form S-nitrosothiols (RSNOs) under both normoxic and hypoxic conditions [61], and a recent study by Bryan et al. demonstrates that steady-state concentrations of tissue nitrite and S-nitroso species are affected by changes in dietary NO<sub>x</sub> (nitrite and nitrate) intake [30•]. Furthermore, enriching dietary intake of nitrite and nitrate translates into significantly less injury from myocardial infarction [44•]. Previous studies demonstrated that nitrite therapy given intravenously prior to reperfusion protects against hepatic and myocardial ischemia/reperfusion (I/R) injury [62]. Additionally, experiments in primates revealed a beneficial effect of long-term application of nitrite on cerebral vasospasm [63]. Moreover, inhalation of nitrite selectively dilates the pulmonary circulation under hypoxic conditions in vivo in sheep [64]. Topical application of nitrite improves skin infections and ulcerations [65]. Furthermore, in the stomach, nitrite-derived NO seems to play an important role in host defense [36] and in regulation of gastric mucosal integrity [66]. All of these studies, along with the observation that nitrite can act as a marker of NOS activity [49], have opened new avenues for the diagnostic and therapeutic applications of nitrite, especially in cardiovascular diseases, using nitrite as a marker as well as an active agent. Oral nitrite has also been shown to reverse L-NAME (NOS inhibitor)-induced hypertension and serve as an alternate source of NO in vivo [67]. In fact, a report by Kleinbongard et al. [68] demonstrates that plasma nitrite levels progressively decrease with increasing cardiovascular risk. Since a substantial portion of steady-state nitrite concentrations in blood and tissue are derived from dietary sources [30•], modulation of nitrate intake along with optimal nitrate-reducing microbial communities may provide a first line of defense for conditions associated with NO insufficiency [50].

**Eradication of Nitrate-Reducing Bacteria Eliminates Health Benefits of Nitrate-Based Diets and Interventions**

In various animal models and in humans, dietary nitrate supplementation has shown numerous beneficial effects, including a reduction in blood pressure, protection against ischemia-reperfusion damage, restoration of NO homeostasis with associated cardioprotection, increased vascular regeneration after chronic ischemia, and a reversal of vascular dysfunction in the elderly [34, 35, 69, 70]. Some of these benefits were reduced or completely prevented when the oral microbiota were abolished with an antiseptic mouthwash [69, 71]. Plasma and salivary nitrite levels are abolished after a dietary nitrate load in healthy subjects taking an antiseptic mouthwash [72]. It has been reported that dietary nitrate reduces blood pressure in healthy volunteers [46, 73], and that the effects are abolished after rinsing with an oral antiseptic mouthwash [74•]. Both strong and weak antibacterial agents suppress the rise in plasma

nitrite observed following the consumption of a high nitrate diet and stronger antiseptics can influence the blood pressure response during low-intensity exercise [75]. Additionally, it was recently shown that in the absence of any dietary modifications, a 7-day period of antiseptic mouthwash treatment to disrupt the oral microbiota reduced both oral and plasma nitrite levels in healthy human volunteers and was associated with a sustained increase in both systolic and diastolic blood pressure [74•]. Oral nitrite exerts antihypertensive effects in the presence of antiseptic mouthwash that disrupts the enterosalivary circulation and reduction of nitrate [76]. Altogether, these studies firmly establish the role for oral nitrate-reducing bacteria in making a physiologically relevant contribution to host nitrite and thus NO levels, with measurable physiological effects. It appears that providing nitrite can overcome the absence of microbial nitrate reduction.

#### Conclusion

Clearly, the potential for the enterosalivary nitrate-nitrite-NO pathway to serve as a NO bioavailability maintenance system by harnessing the nitrate reductase activity of specific commensal bacteria calls for studies that may be profound and truly transformative. These studies will have the potential to (1) redefine the meaning of “healthy oral microbiome” to include microbes associated with NO production, (2) provide a new target for NO-based therapies and open a new direction in cardiovascular research, and (3) allow development of new diagnostic targeted at specific oral microbial communities or select bacteria, the absence of which may reflect a state of NO insufficiency and change the treatment strategies for NO restoration in a number of different diseases.

With the loss of NO signaling and homeostasis being one of the earliest events in the onset and progression of cardiovascular disease, targeting microbial communities early in the process may lead to better preventative interventions in cardiovascular medicine. This may also affect the way oral health professionals recommend oral hygienic practices. Manipulation of the human microbiome as a therapeutic target for disease management is on the near horizon. The oral cavity is an attractive target for probiotic and/or prebiotic therapy because of the ease of access.

A full understanding of the enterosalivary nitrate-nitrite-NO pathway will require the generation and integration of a complete set of data from metagenomic, metatranscriptomic, metaproteomic, and metabolomic studies coupled to biochemical functional assays. The potential to restore the oral flora as a means to provide NO production is a completely new paradigm for NO biochemistry and physiology as well as for cardiovascular medicine and dentistry. There is a known correlation between oral health and systemic disease [77]. Importantly, because oral NO production is dependent on oral nitrate-reducing bacteria, these observations

suggest that the link between oral health issues such as chronic periodontitis and cardiovascular disease may be due in part to decreased abundance of nitrate reducers and concurrent increase of pathogenic bacterial species in the oral cavity. Disruption of nitrite and NO production in the oral cavity may contribute to the oral-systemic link between oral hygiene and cardiovascular risk and disease. The identification of new biomarkers for NO insufficiency and the exploitation of the oral microbiota to increase cardiovascular health will be enabled by further characterization of the enzymatic activities of native oral bacterial communities from larger healthy cohorts and specific patient populations. These cohorts should consist not only of specific US population but also of other around-the-world (European, Asian) populations. It is likely that the oral microbiomes of different ethnic groups, even those within different regions of the US, vary widely. It will be important to determine whether different nitrate-reducing communities are more prevalent in geographically dispersed healthy populations; likewise, it will also be important to determine whether different nitrate-reducing communities are lacking in specific patient populations from around the world. If certain patient populations lack specific nitrate-reducing bacteria, personalized

treatments to enrich  
 fornitratereducersmaybewarranted.Itmaybetimetodiscouragetheuseofantisepticmouthwash.Additi  
 onally,whileantibioticsaresometimes usedtotargetspecificbacterialspecies,itis possible that  
 potential deleterious effects of antibiotic usage on nitrate-reducing communities may preclude the  
 use of antibiotics in specific patient populations. For the past 30 years, scientists have focused on  
 NO production/regulation at the level of nitric oxide synthase (NOS), through the five-electron  
 oxidation of L-arginine. However, this pathway becomes dysfunctional with age and disease [78].  
 The notion that this deficiency can be overcome by targeting oral bacteria is profound and  
 revolutionary. Therapeutically,then,perhapsaneffectivestrategytopromote NO production and  
 overcome conditions of NO insufficiency may not be targeted at eNOS, but rather to target specific  
 oral nitrate-reducingbacterialcommunitiesandincreasingtheconsumption of nitrite and nitrate  
 enriched foods and vegetables. From a public health perspective, we may be able to make better  
 recommendations on diet and relevant host-microbe communities to affect dramatically the  
 incidence and severity of a number of symptoms and diseases characterized by NO insufficiency.  
 Development of novel therapeutics or strategies to restore NO homeostasis can have a profound  
 impact on disease prevention [79]. These new discoveries in the oral bacterial microbiomesuggest  
 thataneffective strategytopromotetherapeuticallyNOproductionandovercomeconditions of NO  
 insufficiency may not solely be to target NOS, but, rather, to focus on understanding specific oral  
 bacterial communities and the optimal conditions for efficient oral nitrate reduction. It appears  
 from early studies that many individuals may not have the optimal microbial communities for Curr  
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maximum nitrate reduction, causing a disruption in a critical NO production pathway.

Understanding and harnessing this alternative pathway may prove to be a viable and  
 costeffectivestrategyformaintainingNOhomeostasisinhumans.

BecauseNOsignalingaffectsallorgansystemsandalmostall  
 diseaseprocessesdescribedtoday,thisnovelapproachtoNO  
 regulationhasthepotentialtoaffectthestudyandtreatmentof many diseases across all organ systems.

Compliance with Ethical Standards

Conflict of Interest Dr. Bryan reports personal fees and other from HumanN, Inc. In addition, he has  
 a patent 8,298,589 with royalties paid to University of Texas, a patent 8,303,995 with royalties paid  
 to University of Texas,apatent8,435,570withroyaltiespaidtoUniversityofTexas,apatent  
 8,962,038withroyaltiespaidtoUniversityofTexas,apatent9,119,823with royalties paid to University  
 of Texas, and a patent 9,241,999 with royalties

paidtoUniversityofTexas.XXXdeclarenofconflictsofinterestrelevantto this manuscript. Drs. Tribble  
 and Angelov declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with  
 human or animal subjects performed by any of the authors.

### 10. Which statement is true:

- a. A 3-Day Food Journal is an excellent way to determine unhealthy food choices.
- b. The ADA has created a dental code for nutritional counseling but can only be used if a dietitian is performing the counseling in the dental office.
- c. Organic wheat bread is a healthy substitute for sourdough bread.
- d. Artificially sweetened sport's drinks are healthy substitutes for cola drinks.

### Barriers and Negative Nudges: Exploring Challenges in Food Journaling

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**Abstract** Although food journaling is understood to be both important and difficult, little work has empirically documented the specific challenges people experience with food journals. We identify key challenges in a qualitative study combining a survey of 141 current and lapsed food journalers with analysis of 5,526 posts in community forums for three mobile food journals. Analyzing themes in this data, we find and discuss barriers to reliable food entry, negative nudges caused by current techniques, and challenges with social features. Our results motivate research exploring a wider range of approaches to food journal design and technology.

**Keywords** Personal Informatics; Food Journals; Barriers; Negative Nudges

### INTRODUCTION

Food journals are an important method for tracking food consumption and can support a variety of goals, including weight loss, healthier food choices, detecting deficiencies, identifying allergies, and determining foods that trigger other symptoms. Although food journaling is widely considered difficult and has motivated research on easing or even automating entry, little work has empirically documented why it is difficult. As a result, the field is currently missing opportunities for design and technology to improve journaling. Our work explores key challenges people encounter in food journaling. We conduct a qualitative study combining a survey of 141 current and lapsed food journalers with analysis

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of 5,526 posts from community forums for three mobile food journals. Analyzing themes in this data, we find and discuss barriers to reliable food entry, negative nudges caused by current techniques, and challenges with social features. Our results support continued research in food journaling, identify specific challenges in current journals, and highlight opportunities to consider a wider range of opportunities in food journal design and technology.

#### RELATED WORK

Recent years have seen significant research in mobile food journals, which aim to improve entry or feedback relative to traditional paper methods. An early example is PmEB, which demonstrated daily calorie budget feedback computed from a mobile food journal [9]. Siek et al. studied journals for patients with chronic kidney disease, who found barcode and audio-based journals overwhelming [7]. Mamykina et al. developed MAHI, a mobile food journal to aid people with newly diagnosed diabetes in developing reflective analysis skills, essential to successful diabetes management [4].

Given the effectiveness of such systems, additional research has considered the adoption, difficulty, and everyday use of food journals. Helander et al. analyze logs from The Eatery, finding only 3% of 190,000 downloads resulted in a person using the mobile food journal for more than one week [3]. PlateMate examines minimizing burden of entry by using crowdsourcing to obtain nutritional summaries from food photos [5]. Rooksby et al. discuss challenges of personal informatics and argue for considering how tracking is intertwined with everyday life [6]. Grimes and Harper suggest that research should consider the variety of relationships people have with food [2]. Despite such interest in how food journals integrate in everyday life, relatively little is known about specific challenges people experience with food journals. We explore current challenges to inform future contributions in food journal design and technology.

#### DATA COLLECTION

We surveyed 141 food journalers (47 active, 94 lapsed, 105 female, 36 male, age 19 to 70, mean 34, median 30), recruited from university mailing lists, MyFitnessPal Facebook groups, and FitBit forums, randomly raffling three \$20 Amazon gift cards. 117 reported journaling with mobile apps, 23 desktop / website, and 47 using paper (some using multiple methods). We also examined 5,526 posts in 627 threads in community forums for three mobile food journals (MyFitnessPal, FatSecret, CalorieCount). We quote survey respondents as P## and attribute forum posts by community.

Survey respondents answered open response questions about what made journaling difficult and what aspects of journaling they disliked. They next used a seven-point Likert scale to rate the difficulty of journaling meal types (buffet meals, ethnic food, fast food, foods served by friends, foods consumed at parties, home-cooked meals, packaged food, restaurant meals). Lapsed journalers additionally provided open responses explaining why they stopped. Responses were analyzed in an affinity diagram exercise by two members of the team, yielding themes later coded by a third.

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We gathered community forum data using provided search interfaces, which allow keyword filtering of threads and posts. We initiated analysis with a set of keyword phrases targeting causes of attrition (e.g., “stop logging” “no fun logging” “not working” “tired of logging”). With these initial search phrases, we found posts such as “It is such a slow and tedious process” (FatSecret), which gave insight into additional keywords to use in searches (e.g., “tedious”). We examined posts using summative content analysis, yielding themes we had not originally specified that were consistent with our survey analysis, as well as an additional theme on challenges in social features of food journals.

**RESULTS AND THEMES Goals Drive Journaling** Journaling can end with a perceived success or at a defined endpoint. Successes included a “goal weight” (P46, P52, P53), understanding eating habits “I had developed a good sense of how many calories are in the different foods I eat and in what proportions I was eating them” (P68), or learning to make healthier food choices “I got a grasp on what to eat and how to eat to maintain optimal health” (P126). Endpoints included identifying a recurring problem “I was trying to track down migraine triggers. Once we identified several triggers, I stopped” (P50) and the end of a pregnancy “I stopped being pregnant:.)” (P82). Of 94 lapsed journalers, only 22 indicated they no longer needed to journal because they received enough benefit. An additional 12 reported periodic journaling: “to audit my consumption” (P85), “whenever I wanted to lose weight” (P51). A few reported journaling with no clear goal: “I wasn’t really trying to lose weight or anything but was simply curious about what I ate. Once I got a decent idea of my eating habits, I didn’t really feel like continuing to journal” (P14).

Although some journalers reach their goals or do not have a specific goal, this is a definite minority. The majority report significant challenges, sometimes leading them to abandon journaling altogether. We discuss these challenges in terms of barriers to reliable food entry, negative nudges in current journals, and challenges with social features.

**Barriers to Reliable Food Entry** Our forum analysis found the effort required to journal is a major barrier: “if I hit a rough spot or start regaining some weight I will go back to counting calories. I hate counting it's tedious but yet very important” (FatSecret). Survey respondents also expressed this, describing food journaling as “too much effort”, “time-consuming”, or “tedious” (31, 27, 16 respondents). Looking more closely, we unpacked this theme into four barriers to reliable food entry.

**What and How Much—**Journalers report challenges knowing what and how much to enter in journals designed around detailed entry. P98 summarizes: “I cook most of my own meals, so it was difficult to estimate the amounts of each ingredient (first, I loosely follow recipes, then I only eat some of what I make at a time), then find the calories (which weren’t available for many of the foods I ate)”. Barriers include ingredients (e.g., “Not knowing the exact ingredients and portions make the logging difficult” P126, 75 others), their

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proportions (e.g., “spaghetti: How much meat? How much oil? Etc...” P107, 10 others), preparation (e.g., “It is also difficult to know how things are cooked sometimes, did they use oil? Does that affect the calories?” P128, 27 others), and then the portion consumed (e.g., “I have a hard time figuring out portions” P23, 39 others). Uncertainty regarding any undermines reliability of an entry.

**Database Reliability**—Many journals use a database to convert entries into calories, other components, or points. These often allow community contribution, with journalers able to publish nutritional entries themselves. Although this can enable a diverse food database, it raises concerns regarding reliability: “the food database is put together by users, so not all entries can be considered accurate” (P32), “sometimes hard to find certain foods, or to know enough about the listing to know if it will be accurate” (P129).

Databases are quite large, but journalers find categories of food missing. From our forum analysis: “Hey all. Any tips on how to record foods not found in database? IE made from scratch, bought at a locally owned restaurant, etc. I'm finding it difficult sometimes to find something close and match it up, or to make a guess” (FatSecret). P2 reported it harder to find healthy foods “They don't have some of the foods I'd like to enter - like more of the healthy and organic foods.” P40 found common foods missing “there are large gaps particularly with foods that are only found in Canada”.

However, continuing to grow the database presents its own barriers. Journalers report that obtaining many results from a query creates concerns for reliability “sometimes it was even incorrect or multiple postings with all different information” (P101), difficulty choosing “there were too many choices and it was difficult to determine which option was the closest to reality” (P105), and frustration with apparently irrelevant entries “I ate some oatmeal and my options for logging it were weird. Oh, was it McDonald's oatmeal? Quaker Instant apple cinnamon oatmeal in a pouch? No. It was normal oatmeal!” (P1). Food journal databases face a tension between providing desired foods versus overwhelming journalers.

**Eating Context**—Journalers report barriers entering food from restaurants, prepared by friends, from buffets, and at parties. Part of the challenge is that knowledge barriers are magnified because the journaler did not prepare the food: “Meals at a restaurant are very difficult because you do not know the ingredients” (P99), “Foods eaten out were tricky due to the ingredients not being clear” (P50), “Calorie databases have some entries for chain restaurants, but finding something that might be similar to a dish at a local restaurant is difficult, if not impossible” (P137).

Another critical challenge in these contexts is people often consume a variety of food over an extended time. P69 felt it was unreasonable to journal: “You're simply not going to sit there with each thing you grab and log it”. This leads to delay, which leads to difficulty remembering: “when I'm eating at a party, or at a buffet I usually eat small amounts of lots of different things. Then when I get back to my apartment and go to log it, I've often forgotten already exactly what I ate, and I'm sure I often miss things.” (P130) and “It was hard to log when I wasn't at home because I had to remember to when I got home” (P112). Despite the always-available nature of mobile devices, contexts in which people eat present

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significant barriers to in-the-moment journaling practices that mobile journals aim to promote.

**Losing the Habit**—Simply forgetting to journal is a major barrier to reliable journals, and was the most-cited reason why survey respondents had missed at least one entry. But journalers further report that missed entries can lead them to abandon journaling altogether. In journals that emphasize calories or other summaries of food components, missing an entry means feedback is inaccurate: “Half of the time, I would just log foods for half the day, so the calories totals were completely off.” (P137). This can in turn create a feedback cycle that undermines the journaling habit: “Every time I start I forget one meal or another so it becomes less accurate. Then I just forget completely.” (P128). P42 noted other factors can lead to breakdowns in her journaling habit “e.g., due to a hard deadline, travel, holiday season”, and reported “once the habit is broken...it’s hard to start again.” Journals cannot assume continuous and complete compliance, and should instead support the journaling habit.

**Negative Nudges in Current Food Journals** Nudges are features of systems that alter behavior [8]. They are typically designed to promote a desired behavior, but we found examples of unintentional negative nudges in current food journals. Journalers report the design of current journals can encourage behaviors contrary to their goals.

**Contrasting Difficulty of Meal Entry**—When asked to rate difficulty by meal type, respondents rated packaged food (average: 6.5) and fast food (6.3) as significantly easier to journal than home-cooked meals (4.6), buffet meals (3.7), ethnic food (3.7), restaurant meals (3.6), foods served by friends (3.2), and foods consumed at parties (2.9) (using a mixedmodel analysis of variance, with participant as a random effect, applying Tukey’s HSD at  $p < .05$ ).

Journalers report databases make packaged and fast food easiest to log, with barcodes further simplifying their entry: “It was easy to search specific brands” (P67), “Fast foods and prepackaged foods have calorie counts readily available” (P86). In contrast, journalers report difficulty entering homemade food: “took too much time, hard to track meals that aren’t pre-packaged” (P43), “a lot of foods are difficult to track without an ingredient list or barcode” (P45).

Many journals provide recipe builders intended to ease the difficulty of entering homemade food. Journalers were aware of this, but still considered it burdensome to enter homemade food: “recipe builders took a long time” (P78), “If a particular item isn’t in my app’s database, I would have to deconstruct it into its constituent ingredients and then add those individually.” (P3), “I often had to search for lots of individual components, and I wasn’t sure of the measurements” (P47). An additional challenge with recipes is that journalers report they do not support variation in preparation: “Even with options to create a meal and save it for later. Most of my cooking isn’t that consistent.” (P101).

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This difficulty contrast creates a dilemma: “Prepackaged meals were the easiest because of bar codes but those aren’t healthy” (P123). This can undermine the journaling habit: “I usually stop [journaling] shortly after I made a wrap or some other type of sandwich that has a significant number of components that it would be difficult to measure and manually

add" (P105). Journalers also reported it can nudge them to eat contrary to their goals, including eating less variety "I could make life easier by eating the same things regularly" (P97), limiting food choice "I just avoided eating things that were hard to log" (P132), and avoiding healthier foods "the time it took entering it manually made eating fresh and healthy less appealing. Easier to scan a code on some processed stuff and be done with it" (P101). Although the mindfulness created by journaling can lead to healthier choices, the difficulty of food entry in current designs is also negatively impacting journaler food choices.

**Judgment and Choosing Not to Journal**—Food journalers report feelings of shame, judgment, or obsession associated with current designs. P6 reported journaling "made me feel guilty sometimes", while P27 noted a lack of positive feedback: "I always felt guilty when I ate too much, and there wasn't that much pride when I was under my goal." Others described not wanting to journal foods they considered unhealthy or that may put them over a calorie goal: "Sometimes I feel like not logging things because I know it's really unhealthy. =( " (P117). Journalers report abandoning journaling due to these feelings: "I stopped because I didn't feel a need to keep it up. I did not want to obsess about food" (P13), "it made me too focused and obsessive about what I was eating" (P70), "I think I'm obsessing about every calorie and I'm either not eating enough or eating too much" (MyFitnessPal). P113 was especially concerned: "it was more of an on the way to an eating disorder thing than anything else (tried to keep calories extremely low)". Although in-the-moment feedback can be powerful, designs that create feelings of judgment can be selfdefeating.

**Stigma and Journaling**—Journaling often takes place in the presence of others, and P37 noted "it's not always that discreet". Many did not want friends and colleagues to know they were tracking food "I think I was hesitant to do the logging if not alone" (P30) and were afraid to ask the ingredients in food "I had more of a problem with eating out at a friend's house because I didn't want to ask for ingredients or mention that I was logging calories" (P11). This can lead people to abandon journaling: "I also felt embarrassed to do it in front of friends so I stopped" (P27). Journalers also report being nudged to avoid social situations: "It discourages you from eating out or at a friend's, even if it is healthy" (P42). Journalers can struggle with a perceived stigma around tracking, undermining not only the reliability of their journal but also their goals and motivation.

**Challenges in Social Features** The previous challenges emerged in both datasets, but our community forum analysis additionally surfaced challenges in provided social features. Journalers turn to these for social support "please if you want to motivate me and help me out with comments and suggestions, add me as a friend" (MyFitnessPal), and some find that support "I have found that having friends on MFP has helped me, just having someone say good job when I do my

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exercise or finish under my calorie goals" (MyFitnessPal). But others find support never materializes "I have seen lately that I will post my diary or my progress or even some troubles and no one will comment back. How is that going to encourage me to keep going when I have no support" (MyFitnessPal), that their motivation is undermined as friends

stop journaling “Although I used to log frequently in the past, most of my connections have stopped logging in or are no longer members, so I recently emptied my ‘buddy list’” (FatSecret), or they are discouraged by the success of others “I hate coming on to forums and seeing how much people have lost and I have made barely any progress at all.” (FatSecret). Embarrassment leads people to omit entries or disable sharing: “I have logged food on here before but I get embarrassed so I made it private so only I can see and told myself be honest with yourself” (MyFitnessPal). Social features can both support or undermine journalers, and need to be carefully considered.

#### DISCUSSION AND CONCLUSION

We have contributed a qualitative analysis of two datasets to empirically document specific challenges people encounter in food journaling. By unpacking “too much effort” into specific challenges, our findings suggest opportunities to improve food journaling design and technology.

One important opportunity is to consider journaler goals and how they relate to these challenges. For example, detailed nutritional data may be important to a journaler pursuing weight loss. But the challenges in obtaining that data might be inappropriate for a journaler looking to identify a food trigger or gain a higher-level understanding of food habits (e.g., for whom “oatmeal” is sufficient). Conversely, details of preparation might be irrelevant to a journaler interested only in calories but critical to identifying a food trigger. Instead of attempting to capture the elusive “everything”, these findings suggest a diversity of journal designs to support specific goals.

Challenges with food databases suggest several opportunities. A reputation system might address reliability of community entries, and could consider goals (e.g., a journaler tracking sodium intake may find an entry more reliable if created by another journaler with a similar goal). Further, a community could vote on the accuracy of a contributed entry (e.g. “6 of 8 journalers agree with this assessment of calories”). The tension between databases providing desired foods but overwhelming journalers might be addressed using context (e.g., surface likely food at a particular restaurant), personal diets (e.g., a vegetarian’s journal), routines (e.g., a person may always have the same espresso drink), or designs for lower-fidelity journals (e.g., using nutritional information from a generic “oatmeal” entry instead of strictly requiring greater disambiguation for a minor nutritional difference).

One initial promising opportunity we have pursued is a photo-based food journal, which can level the challenge of journaling different meal types, provide value in spite of missing entries, and avoid creating judgment by not emphasizing nutritional details [1]. But our findings point out a variety of challenges in food journaling and motivate future research exploring how new designs and technology can support journalers in their food-related goals.

### 11. Which statement is false:

- a. Water pick devices are always effective when used in deep pockets at high pressure.
- b. Interproximal cleaning brushes like Tepe and GUM are effective in removing unhealthy dental plaque.
- c. If any bleeding occurs when using Tepe or GUM interproximal brushes, then this is most often a sign of active gum disease.
- d. Published medical research has shown that you should wait at least an hour before brushing after drinking an acid drink.

#### An Overview of Different Interdental Cleaning Aids and Their Effectiveness

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**Abstract:** Optimisation of plaque control is essential for the success of non-surgical and surgical periodontal therapy.

This cannot be achieved with brushing alone; hence, there is a need for adjunctive interdental cleaning aids. The aim of this paper is to provide an overview of different interdental cleaning aids and review the literature for consensus on their effectiveness. A literature search of articles in English, up to December 2018, was conducted in Pubmed. High-quality flossing is difficult to achieve, and ineffective routine use of floss may not confer significant benefits over brushing alone.

Interdental brushes are more effective than brushing as a monotherapy.

They are at least as good if not superior to floss in reducing plaque and gingivitis.

Although they are effective for patients regardless of their periodontal status (healthy or active), they are especially indicated in periodontal patients where widened embrasures are common. Added benefits include ease of use, patient acceptance, and recontouring of interdental tissues. Rubberpicks do not demonstrate inferiority to conventional interdental brushes. Wooden interdental aids appear to offer no significant advantage over brushing with respect to plaque removal; they may, however, reduce gingival bleeding. Oral irrigators are a promising tool for reducing gingival inflammation, despite minimal changes to plaque levels. For cleaning around dental implants, oral irrigators and interdental brushes are preferred over floss.

**Keywords:** dental devices; interdental; oral health; dental plaque

1. Introduction A patient's ability to achieve good mechanical plaque control is vitally important. Scaling and root planing without effective plaque control during the healing/maintenance phase results in subgingival recolonisation within 4–8 weeks [1]. Conversely, good supragingival plaque control appears to be sufficient in preventing relapse or recurrence of the disease due to subgingival recolonization [2]. Poor oral hygiene is also a significant risk factor for unsuccessful periodontal surgery [3], stability of guided tissue regeneration results [4], and peri-implant disease [5]. Today, although toothbrushing is the most

common method of mechanical plaque removal, we may still not be very good at it. A systematic review by van der Weijden et al. found that, in adults with gingivitis, self-performed mechanical plaque removal with a manual toothbrush was not sufficiently effective [6]. More frequent tooth cleaning (up to twice daily) was shown to significantly improve gingival health [7]. The reality is that brushing alone may only remove up to 60% of overall plaque at each episode of cleaning [8]. A more recent systematic review by Slot et al. estimated that the efficacy of plaque removal following a brushing exercise averages around 42% [9]. Brushing is also thought to be more optimal for cleaning facial surfaces of teeth compared to interproximal surfaces [10]. This is significant because interdental sites present the highest risk of plaque accumulation, whether anteriorly or posteriorly in the mouth [11].

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Thus, interproximal surfaces of molars and premolars, being the predominant sites of residual plaque, are at higher risk of developing periodontal lesions and caries [12,13]. Clinically, gingivitis and periodontitis are usually more pronounced in interproximal areas than facial aspects [13].

Carefully performed supragingival plaque control was shown to be capable of altering the quantity and composition of both supragingival and subgingival microbiota.

This was demonstrated by Dahlen et al., who found that, 24 months after initiation of a supervised oral hygiene program, total viable counts of bacteria in deep and shallow pockets and important bacteria species such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* decreased in number [14]. To aid in plaque control, various interdental cleaning aids are used. These include dental floss, interdental brushes, wooden interdental aids, and oral irrigators. A recent study by Marchesan et al. provides convincing data to support the use of interdental cleaning devices for promoting good oral health outcomes. The study found that interdental cleaning is associated with less periodontal disease, less coronal and interproximal caries, and fewer missing teeth; a higher frequency of interdental cleaning (4–7 times per week) was also associated with less interproximal periodontal disease [15]. These findings are in agreement with Crocombe et al., who found that regular interdental cleaning was associated with less plaque, calculus, and gingivitis [16]. The aim of this paper is to provide an overview of different interdental cleaning aids and review the literature for consensus on their effectiveness.

**2. Materials and Methods** A literature search was conducted in Pubmed using the Pubmed clinical query tool in December 2018.

The keywords (interdental) AND (cleaning) were used, together with filters on category (therapy) and scope (broad). The search yielded 105 articles published between 1974 and 2018.

Related articles in English, including reviews, meta-analyses, and clinical trials in humans, were included. Studies utilising interventions other than interdental cleaning aids (e.g., mouth rinses or subgingival irrigation), new products not commonly available in the market, and orthodontic subject populations were excluded. Abstracts were screened for relevance and full text was assessed for relevant articles. A manual search of the reference section of retrieved articles was also performed.

**3. Dental Floss** Routine use of dental floss is low, ranging between 10% and 30% among adults [17]. The low compliance observed among adults could be because flossing is a technically challenging task. Studies showed that few individuals floss correctly and

patients find flossing difficult, especially in areas with tight contact points [18–20]. Consequently, it was found that unsupervised flossing does not result in substantial reductions in gingival inflammation [21].

There is some evidence in the literature that the use of floss as an adjunct to brushing is potentially ineffective (Table 1). A review conducted by Berchier et al. concluded that routine instruction to use floss is not supported by evidence [22]. The results of a Cochrane review conducted in 2011 found “weak and very unreliable evidence” that flossing as an adjunct to brushing may be associated with a small reduction in plaque, although they did note a significant benefit with reducing gingivitis [23]. These findings are consistent with a meta-review in 2015, which states that most available studies fail to demonstrate the effectiveness of flossing in plaque removal, potentially due to technical difficulty or lack of patient compliance [24]. Indeed, adjunctive use of floss did not contribute further to plaque reduction compared to toothbrushing alone, even in young patients with intact papilla [25,26]. Despite substantial evidence citing a lack of support for the effectiveness of flossing in plaque removal, flossing may still confer benefits. Flossing itself is not harmful and has no major associated health risks, in addition to occasional short-term soft-tissue trauma [27]. Professional flossing was shown to be effective in reducing interproximal caries risk; however, this beneficial effect was lost when flossing was self-performed [28].

For patients lacking dexterity or compliance, floss holders represent a potential alternative. Studies demonstrated similar effectiveness of floss holders compared to handheld floss in reducing interproximal plaque and gingivitis [29,30].

They may also benefit patients lacking the  
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dexterity to use hand floss [29].

Further, floss holders are significantly more effective in helping patients establish a long-term flossing habit, with floss holder users more likely to floss than hand-flossers [31]. It should be noted, however, that these studies comparing floss with floss holders did not include a control group for brushing. Reductions in interproximal plaque and gingivitis from pre-treatment levels are reasonable expectations, and this may not have differed when comparing the results to a group using brushing alone. Hence, most studies do not support the routine use of floss. Reductions in plaque or gingivitis can only be expected if patients can achieve high-quality flossing regularly. However, this may be an unrealistic expectation.

Table 1. Summary of included studies on the effectiveness of flossing

Study	Study Design and Evaluation Period	Intervention	Primary Outcome Measures
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Berchier et al. [22]			
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	Systematic review, 11 studies, 559 subjects, 1–6 months		
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		Dental floss as an adjunct to brushing	Plaque index, gingival index
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		Flossing provides no additional benefit to brushing for the removal of plaque and reduction of gingivitis	
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Sambunjak et al. [23]			
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	Systematic review, 12 studies, 1083 subjects, 1–6 months		
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		Dental floss as an adjunct to brushing	Plaque index, gingival index
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Significant benefit associated with flossing as an adjunct in reducing gingivitis. Insufficient evidence for reducing plaque

Kiger et al. [26]

Crossover study, 30 previously treated periodontal subjects, 3 months

Brushing vs. brushing + floss or interdental brushes as an adjunct

Gingival index, proximal plaque scores, buccal and lingual plaque index

The use of floss as an adjunct did not result in significant differences for any of the parameters

Graziani et al. [25]

Randomised clinical trial, 60 periodontally healthy subjects, 1 month

Brushing vs. brushing + floss or interdental brushes or rubber interdental brushes

Full-mouth bleeding scores, full-mouth plaque scores, probing pocket depth, recession, angulated bleeding score

The use of floss did not result in significant benefits over brushing alone

Blanck et al. [30]

Two-phase crossover study, 26 subjects, study duration not mentioned

Floss vs. floss holder Plaque index

Both products removed statistically significant amounts of plaque compared to pre-treatment levels

Kleber et al. [31]

Survey, 32 respondents, 6 months after completion of a flossing study

Floss vs. floss holder

Number of participants flossing regularly and with which method after study completion

Floss holders are more effective in helping patients establish a regular flossing habit

Pucher et al. [29]

Comparative study, 36 dental students and 26 adult patients undergoing periodontal maintenance, 6 weeks

Floss vs. floss holder Plaque index, gingival index

Both methods are effective in reducing interproximal plaque and gingivitis

#### 4. Interdental Brushes (IDBs)

##### 4.1. Adjunctive Use of Interdental Brushes

Interdental brushes were investigated as early as 1976, where it was found that they were effective in removing plaque as far as 2–2.5 mm below the gingival margin [32]. They consist of a central metal wire core, with soft nylon filaments twisted around. The effectiveness of interdental brushes is well documented. One of the consensus findings from the European Federation of Periodontology 2015 workshop states that “cleaning with interdental brushes is the most effective method for interproximal plaque removal, consistently associated with more plaque removal than flossing or woodsticks” [33]. Two systematic reviews found that the adjunctive use of interdental brushes results in significant improvements on clinical parameters such as plaque scores, bleeding scores, and probing depth, when compared to brushing alone [34,35]. Another review by Salzer et al. found that interdental brushes were the most effective method for interdental plaque removal, compared to other interdental cleaning aids [24]. The superiority of interdental brushes is thought to be due to higher efficacy of

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plaque removal and high patient acceptance, as well as ease of use [10,26,33,36]. Thus, it is clear that the use of interdental brushes as an adjunct provides a clinical benefit over brushing alone.

**4.2. Choosing an Appropriate Interdental Brush** There are several factors to consider when choosing an interdental brush which may affect its efficacy. The first is that size matters. In 2011, Imai et al. conducted a study which used a measuring tool to determine the best-fitting interdental brush for proximal sites. A colour-coded probe was inserted horizontally from the buccal aspect until snug, with the resulting colour on the probe corresponding to the matching colour of the interdental brush.

The reduced number of bleeding sites noted in this study was attributed to the use of an appropriate size of interdental brush, which resulted in "effective disruption of proximal biofilm compared to one size for all proximal spaces" [36]. Bourgeois et al. also used a colour-coded probe to determine the appropriate size of interdental brush. They found that calibrated interdental brushes reduce interdental bleeding by 46% at one week and 72% at three months [37]. These findings are consistent with the opinion of other authors, who observed that the size of the interdental brush should fit snugly in the interdental space [34]. Most studies neither discussed interdental brush sizes used, nor indicated if interproximal brushes were used at all available proximal sites [34]. Failure to use an appropriate size of interdental brush may account for the lack of statistical difference between various interdental cleaning aids in other studies [36]. Another factor to consider is the geometry of the interdental brush. Straight interdental brushes may be more effective in interproximal plaque removal than angled interdental brushes with a long handle. Findings from Jordan et al. 2014 showed that straight interdental brushes were statistically superior at plaque removal than angled brushes; they were also found to be significantly more effective for posterior teeth [38]. Waist-shaped interdental brush heads were also found to remove significantly more biofilm than straight interdental brushes, resulting in lower plaque scores [39]. This can be explained by the superior cleansing effect on buccal and lingual line angles of waist-shaped brushes [39].

A triangular or conical form of interdental brushes may also provide better adaptation to the interdental space [34]. The material of the interdental brush is also worthy of consideration. It was observed that the metal wire in the middle of interdental brushes can be uncomfortable for patients with sensitive root surfaces [40]. Rubber interdental brushes/picks are a more recent development and could be a viable alternative to conventional interdental brushes. They were shown to be as effective as regular interdental brushes (metal core), with further benefits of greater patient compliance and acceptance, in terms of comfort and willingness to buy the product [25,41,42]. Regarding the safety profile of interdental brushes, there is concern regarding their use at healthy sites with no attachment loss, potentially resulting in trauma [33]. Existing studies, however, did not find any associated gingival damage [38,43] or hard-tissue damage after the use of interdental brushes [43]. No attachment loss was found for patients using interdental brushes for over ten years [32]. It should be noted that the interdental papilla is pressed downward while achieving its subgingival cleaning action [44]. This may help in recontouring interdental tissues, although there may be associated recession.

4.3. Floss vs. Interdental Brushes Many studies investigated the use of interdental brushes compared with floss using various clinical parameters (Table 2). In periodontally healthy subjects, the adjunctive use of interdental brushes or rubber interdental picks resulted in lower interdental plaque scores compared to brushing alone; the same was not true in the group that used floss as an adjunct [25]. Interdental brushes were also found to be superior in reducing interproximal bleeding in healthy patients with filled embrasures [36]. In a population of patients with mild to moderate chronic periodontitis, bleeding on probing and probing depth were reduced over a month of follow-up when interdental brushes, but not floss, were used [45]. This study found that interdental brushes and floss did not differ statistically for removal of supragingival or subgingival plaque. Christou et al. investigated the use of interdental brushes vs. floss in untreated patients with moderate to severe periodontitis. Although they found no difference between floss and interdental brushes for bleeding indices, interdental brushes were associated with more effective plaque removal and greater pocket reduction [10]. These findings are consistent with another study conducted using a population of untreated chronic periodontitis patients. Changes in plaque, papillae level, and probing depths were found to be significantly greater in the interdental brush group than the floss group [44]. The superiority of interdental brushes over floss is also apparent in patients undergoing periodontal maintenance. This was demonstrated by two studies, which showed that interdental brushes (IDBs), when used as an adjunct to toothbrushing, are more effective in proximal plaque removal than floss [26,46]. However, not all the literature is in unanimous agreement. In 2013, a Cochrane review concluded that, although there is some evidence that IDBs reduce gingivitis at one month compared to flossing, there was insufficient evidence to claim additional benefits of interdental brushes as an adjunct for reducing plaque [35]. Thus, it can be surmised from existing evidence that, regardless of the periodontal status of the patient, interdental brushes appear to be superior (whether based on gingival index, plaque index, reduction in bleeding or probing, reduction in probing depths, or a combination) to floss. The use of interdental brushes for the removal of proximal plaque seems to be especially indicated in periodontal patients or patients on a maintenance program [10,26,44,46]. This could be because the bristles of an interdental brush are able to fill embrasures better and engage exposed irregularities in root surfaces. Another clinical situation where interdental brushes may be superior is cleaning around implants. Peri-implantitis was shown to be associated with inadequate plaque control and prosthetic constructions that did not allow accessibility to oral hygiene measures. Similar to some studies in natural teeth, interdental brushes demonstrated greater efficacy in removing proximal biofilm around implants [47]. The application of floss to exposed rough surfaces of an implant may also lead to fraying and, ultimately, the development of peri-implant conditions; hence, using interproximal brushes or toothpicks may be preferable [48]. With an increase in the number of implants placed and the occurrence of peri-mucositis and implantitis, effective oral hygiene to remove biofilm is likely to play an important role in future clinical practice.

Table 2. Summary of included studies on the effectiveness of interdental brushes and rubberpiks.

Study	Study Design and Evaluation Period	Intervention	Primary Outcome Measures	Conclusion
Imai et al. [36]	Randomised clinical trial, split mouth, 30 subjects, 12 weeks	Floss vs. IDB	Plaqueindex,bleedingindex	No difference between both methods for plaque removal. IDBs are superior for reducing bleeding
Noorlin et al. [45]	Clinical trial, split mouth, 10 subjects with untreated periodontal disease, 1 month	Floss vs. IDB	Approximal plaque scores, bleeding on probing, probing depth	Both devices result in similar beneficial effects on plaque scores. Bleeding and mean probing depth reduction was significantly reduced compared to floss sites
Christou et al. [10]	Randomised clinical trial, split mouth, 26 subjects with untreated periodontal disease, 6 weeks	Floss vs. IDB	Plaque scores, probing depth, periodontal pocket bleeding index, angulated bleeding index	No difference between floss and IDB for bleeding indices. IDBs are more effective in plaque removal
Jackson et al. [44]	Randomised clinical trial, 77 subjects with untreated periodontal disease, 12 weeks	Floss vs. IDB	Plaque index, bleeding index, bleeding on probing at interdental sites, relative interdental papillae level	More improvements in the IDB group for every parameter
Kiger et al. [26]	Crossover study, 30 previously treated periodontal subjects, 3 months	Brushing only vs. brushing + floss or IDB as adjunct	Gingival index, proximal plaque scores, buccal and lingual plaque index	IDBs as an adjunct are superior to floss for proximal plaque removal
Rosing et al. [46]	Comparative study, 55 subjects on a maintenance program, no follow-up period	Floss vs. IDB	Plaque index	IDBs are superior to floss for removal of interdental plaque
Graziani et al. [25]	Randomised clinical trial, 60 periodontally healthy subjects, 1 month	Brushing vs. brushing + floss or interdental brushes or rubber interdental brushes	Full-mouth bleeding scores, full-mouth plaque scores, probing pocket depth, recession, angulated bleeding score	Adjunctive use of IDBs or rubber picks reduces more interdental plaque than brushing alone or flossing

Study Study Design and Evaluation Period Intervention Primary Outcome Measures  
Conclusion

Hennequin-Hoenderdos et al. [42]

Randomised clinical trial, split-mouth, 42 subjects, 1 month

Conventional IDBs vs. rubber bristles

Bleeding on marginal probing, plaque index, gingival abrasion score

Rubber bristles were more effective in reducing gingival inflammation. They were associated with less gingival abrasion and better received by participants

Bourgeois et al. [37] Randomised clinical trial, 46 subjects, 3 months IDBs vs. brushing  
Frequency of interdental bleeding

IDBs resulted in significantly less interdental bleeding (plaque scores not evaluated)

Abouassi et al. [41]

Randomised clinical trial, crossover design, 39 subjects, 1 month

Conventional IDBs vs. rubber bristles

Plaque index, bleeding index, patient satisfaction questionnaire

Rubber bristles were as effective as conventional IDBs with the added benefit of more comfort

Jordan et al. [38] Randomised clinical trial, 128 subjects, 12 days

Straight vs. angled IDBs

Modified proximal plaque index

Straight IDBs were better at removing interproximal plaque, especially for posterior teeth

Chongcharoen et al. [39]

Randomised clinical trial + crossover study, 8 subjects post-initial periodontal therapy, 3 weeks

Straight vs. waist-shaped IDBs Plaque index Waist-shaped IDB had lower plaque scores than straight IDBs

Slot et al. [34] Systematic review, 9 studies, 510 subjects, 1–3 months

IDBs as an adjunct to brushing

Plaque index, gingival index, bleeding on probing, probing depth

IDBs as an adjunct to brushing showed significant reductions in plaque scores, bleeding scores, and probing depth

Poklepovic et al. [35] Systematic review, 7 studies, 354 subjects, 1–3 months

IDBs and flossing as adjuncts to brushing Gingival index, plaque index

Adjunctive use of IDBs results in significant improvements in gingival and plaque indexes compared to brushing alone. There is some evidence that IDBs reduce gingivitis at 1 month compared to flossing. More evidence needed for 3 months. No conclusions could be drawn for a difference for plaque index

Luz et al. [47] Clinical trial, crossover study, 12 subjects, 2 months

Floss vs. IDB (around implants) Plaque index IDBs are more efficacious in removing proximal biofilm around implants

5. Wooden Interdental Aids Woodsticks are designed for mechanical removal of proximal plaque, achieved by friction against proximal tooth surfaces [40]. Comparatively fewer studies investigating woodsticks or toothpicks exist (Table 3). Similar to interdental brushes, woodsticks are able to remove plaque up to 2–3 mm subgingivally by depressing the papilla [49]. They fit best into interdental spaces with a triangular cross-section and should not be confused with toothpicks. Toothpicks are round, allowing only point contact

with the tooth surface and, thus, are more suited for removing food debris after a meal [18].

One study, however, demonstrated similar efficacy of supragingival plaque removal to woodsticks [50]. A systematic review of seven studies observed that woodsticks do not have an additional benefit on proximal plaque reduction compared to toothbrushing alone, although a reduction in gingival bleeding scores was noted [51].

Four of these studies compared the efficacy of woodsticks to floss, with three finding no significant difference in plaque scores and one favouring the use of floss. This result is similar to another early study which reported a comparable proximal plaque removal efficacy between toothpicks and floss [52]. Woodsticks may specifically remove subgingival plaque that is not visible; hence, gingival inflammation parameters may improve while minimally affecting the plaque index [51]. Indeed, Finkelstein et al. observed that, by disrupting interdental plaque, interdental cleaning aids can result in significant reduction in gingival inflammation, although they may appear to have minimal effects on visible tooth surface plaque accumulation [53]. Like interdental brushes, the use of woodsticks may require a certain amount of interdental space to be present. They seem the most appropriate for open interdental spaces in regions that are not too posterior in the mouth, due to their specific angle of entry into the embrasure [54]. The advantages of toothpicks/woodsticks include ease of use and convenience.

They may be more acceptable to older patients, especially those who routinely use toothpicks to remove food debris after eating.

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Table 3. Summary of included studies on the use of woodsticks and toothpicks.

Study	Study Design and Evaluation Period	Intervention	Primary Outcome Measures
Hoenderdos et al. [51]	Systematic review, 7 studies, 438 subjects, 3–14 weeks	Woodsticks as an adjunct to brushing	Plaque index, gingival index
No significant advantage on plaque removal, but reduces gingival bleeding tendency			

Schmid et al. [52]

Clinical trial, crossover study, 21 periodontally healthy subjects, 2 weeks

Toothbrush vs. floss vs. toothpicks

Plaque index

No differences for visible proximal surfaces. Brushing was superior on buccal surfaces.

Toothpicks were as efficient as brushing on lingual surfaces

Zanatta et al. [50] Clinical trial, split mouth, 15 subjects

Toothpicks vs. woodsticks

Plaque index

No differences found between round toothpicks and triangular woodsticks for

supragingival plaque removal

6. Oral Irrigators Oral irrigators were first introduced to the dental profession in 1962 [55]. In 2001, the American Academy of Periodontology stated that “supragingival irrigation with or without medicaments reduces gingival inflammation beyond what is normally achieved by toothbrushing alone” [56].

The mechanical mode of action of oral irrigators is through a combination of pulsation and pressure. This provides phases of compression and decompression of gingival tissue, removing supragingival plaque and flushing out subgingival bacteria and other debris [57]. Two zones of hydrokinetic activity are created—an impact zone where the solution contacts the gingival margin, and a flushing zone where the irrigant reaches subgingivally [55].

In addition to their ability to flush away loosely adherent plaque, remove bacteria cells, and interfere with plaque maturation [58],

the use of oral irrigators was shown to reduce inflammation by reducing pro-inflammatory cytokines (IL-1 $\beta$  and PGE2) in the gingival crevicular fluid of patients with localised mild to moderate periodontitis and diabetics [59,60]. Indeed, by altering specific host-microbial interactions in the subgingival environment, pulsations from oral irrigators may reduce inflammation independent of plaque removal [61]. Cobb et al. observed that oral irrigators facilitate removal of subgingival biofilm up to 6mm, inducing qualitative changes in subgingival plaque while not harming soft tissue [62]. The depth of penetration of oral irrigation was also measured in other studies. Indeed, 90% pocket penetration was achieved when probing depths were  $\leq 6$  mm [63]; oral irrigators were found to penetrate on average about half the depth of pockets, with the greatest penetration for pockets 0–3mm and  $>7$ mm [64]. The use of an oral irrigator does not appear to require any special motor skill, as demonstrated in 5.5–6.5-year-old children [65].

Attached gingiva can withstand pressure up to 160psi for 30s without irreversible damage, leading Bhaskar et al. to recommend that 90 psi is acceptable on undamaged tissue and 50–70 psi suitable for inflamed or ulcerated tissue [57]. A position paper by the American Academy of Periodontology supports the use of supragingival irrigation at forces of 80–90 psi, also commenting that oral irrigation poses no safety hazard as bacteraemia levels are similar to toothbrushing, flossing, scaling, and chewing [66]. Information concerning higher-risk patients who require prophylaxis prior to periodontal therapy is unavailable. Several studies exist supporting the efficacy of oral irrigators as compared with other interdental devices (Table 4). Most of the studies were generated using commercially available products like Waterpik and Philips Sonicare Airfloss. Most of the studies demonstrated that oral irrigators may be more effective than dental floss or interdental brushes in reducing bleeding, plaque, or probing depths [59,60,67–75]. The utility of oral irrigators can also be extended to implant maintenance as shown by Magnuson et al., who observed that the water flosser group had significantly greater bleeding reduction than floss cleaning around implants [76].

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A systematic review of seven included studies published between 1971 to 2000 showed that oral irrigation does not have a beneficial effect in reducing visible plaque, although there is a trend favouring improvement in gingival health compared to toothbrushing alone [77]. Indeed, some studies discussed showed improvement in plaque index parameters while others did not. Secondary subgingival penetration after supragingival irrigation may explain why gingival inflammation is frequently diminished, despite unchanged plaque levels [66]. Thus, the benefit of oral irrigators seems to be reducing parameters of gingivitis, and this effect may not be related to plaque removal.

Table 4. Summary of included studies on oral irrigators.

Study	Study Design and Evaluation Period	Intervention	Primary Outcome Measures
Cutler et al. [59]	Clinical trial, crossover study, 52 subjects, 2 weeks	Oral irrigator vs. brushing	

Probing pocket depth, bleeding on probing, gingival index, plaque index, IL-1 $\beta$  and PGE2 levels

Improvement in all clinical parameters with oral irrigation. A reduction in pro-inflammatory cytokine profile in gingival crevicular fluid

Al-Mubarak et al. [60]

Randomised clinical trial, 52 diabetic subjects with adult periodontitis, 12 weeks

Oral irrigator vs. brushing

Modified gingival index, probing pocket depth, plaque index, clinical attachment level, bleeding on probing, reactive oxygen species generation, cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-10, PGE2), and HbA1c

Significant reduction in plaque index, gingival index, bleeding on probing, and reactive oxygen species generation compared to brushing. Significant reduction in IL-1 $\beta$ , and PGE2 from baseline within test group

Newman et al. [68]

Multi-centre randomised clinical trial, 155 subjects receiving supportive periodontal therapy, 6 months

Regular oral hygiene vs. oral irrigation with water vs. oral irrigation with water and zinc sulphate solution

Bacterial measurements, gingival index, bleeding on probing

Oral irrigation with water was superior to regular oral hygiene and additional irrigation with zinc sulphate for reducing gingival inflammation. Oral irrigation with water significantly reduced bleeding on probing compared to regular oral hygiene

Barnes et al. [69] Randomised clinical trial, 105 subjects, 1 month

Manual toothbrush + floss vs. manual toothbrush + oral irrigator vs. sonic toothbrush + oral irrigator

Bleeding index, gingival index, plaque index

Irrigation groups were more effective in reducing bleeding index and gingival index. The manual toothbrush + floss group was less effective than the sonic toothbrush + irrigation for reducing plaque

Rosema et al. [75]

Randomised home-use experiment, 108 subjects, 1 month

Oral irrigator vs. floss Bleeding index, plaque index Oral irrigator is superior to floss in reducing gingival bleeding

Goyal et al. [70] Randomised clinical trial, 70 subjects, single use Oral irrigator vs. floss Plaque index Oral irrigator as adjunct is superior to floss as in plaque removal

Husseini et al. [77] Systematic review, 7 studies, 590 subjects, 2–6 months Oral irrigators vs. brushing

Plaque index, bleeding index, gingival index, probing pocket depth

Oral irrigators do not have a beneficial effect in reducing visible plaque. However, there is a trend of improving gingival health by reducing bleeding

Goyal et al. [71] Randomised clinical pilot study, 28 subjects, two weeks Oral irrigator vs. IDBs Plaque index, bleeding on probing

Oral irrigators are more effective than IDBs for reducing gingival bleeding. No difference for plaque index

Lyle et al. [73] Randomised pilot study, 28 subjects, single-use Oral irrigator vs. IDBs Plaque index

Oral irrigators as adjuncts remove significantly more plaque than IDBs after a single use  
 Stauff et al. [67] Randomised clinical trial, 60 subjects, 4 weeks  
 Phillips Airfloss (microdroplet device) vs. floss  
 Papilla bleeding index, modified proximal plaque index, amount of gingival crevicular fluid  
 Microdroplet device was more effective at reducing plaque, with the added benefit of comfort of use  
 Goyal et al. [72] Randomised clinical trial, 69 subjects, 1 month  
 Waterpik vs. Sonicare Air Floss Pro  
 Bleeding on probing, modified gingival index, plaque index  
 Waterpik is significantly more effective than Sonicare in reducing bleeding and gingivitis  
 Sharma et al. [74] Randomised clinical trial, 82 subjects, single-use  
 Waterpik vs. Sonicare Airfloss Plaque index  
 Waterpik removes significantly more plaque from tooth surfaces than Sonicare  
 Magnuson et al. [76] Randomised clinical trial, 40 implants, 1 month  
 Oral irrigator vs. floss (around implants) Bleeding on probing  
 Oral irrigator demonstrated significantly greater reduction in bleeding than floss

## 7. Summary

Interdental cleaning aids play a vital role in optimising gingival health and preventing oral disease. Based on the results of this review, interdental brushes provide a significant benefit over brushing as a monotherapy. The use of floss may not achieve similar results if not effectively performed. Regarding gingival and plaque indices, interdental brushes may be superior to dental floss in at least one

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parameter, with the added benefits of patient comfort and acceptance. They are especially indicated in periodontal patients, who are likely to have widened embrasures. Rubberpiks were shown to be comparable to interdental brushes for reducing gingivitis and plaque. The biphasic mode of action of oral irrigators may result in qualitative changes in subgingival plaque. They may, thus, reduce gingival inflammation, although overall plaque levels as measured supragingivally may appear unaffected. Wooden interdental aids appear to offer no significant reduction in plaque compared to brushing, although they may reduce gingival inflammation.

Interdental brushes and oral irrigators are recommended over floss for implant maintenance. We provided an overview of different interdental cleaning aids and their effectiveness. However, there is no single cleaning aid that works best for all patients. The option of an appropriate interdental cleaning aid is also influenced by the ease of use, size of interdental space, acceptability, dexterity, and motivation of the individual.

Most periodontal disease arises from, or is aggravated by, accumulation of plaque, and periodontitis is associated particularly with anaerobes such as *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Actinobacillus actinomycetemcomitans*. Calculus (tartar) may form from calcification of plaque above or below the gum line, and the plaque that collects on calculus exacerbates the inflammation. The inflammatory reaction is associated with progressive loss of periodontal ligament and alveolar bone and, eventually, with mobility and loss of teeth. Periodontal diseases are ecogenetic in the sense that, in subjects rendered susceptible by genetic or environmental factors (such as polymorphisms in the gene for

interleukin 1, cigarette smoking, immune depression, and diabetes), the infection leads to more rapidly progressive disease. Osteoporosis also seems to have some effect on periodontal bone loss. The possible effects of periodontal disease on systemic health, via pro-inflammatory cytokines, have been the focus of much attention. Studies to test the strength of associations with atherosclerosis, hypertension, coronary heart disease, cerebrovascular disease, and low birth weight, and any effects on diabetic control, are ongoing.

**Gingivitis** Chronic gingivitis to some degree affects over 90% of the population. If treated, the prognosis is good, but otherwise it may progress to periodontitis and tooth mobility and loss. Marginal gingivitis is painless but may manifest with bleeding from the gingival crevice, particularly when brushing the teeth. The gingival margins are slightly red and swollen, eventually with mild gingival hyperplasia. **Management**—Unless plaque is assiduously removed and kept under control by tooth brushing and flossing and, where necessary, by removal of calculus by scaling and polishing by dental staff, the condition will recur. Although gingivitis has a bacterial component, systemic antimicrobials have only transient benefit and therefore no place in treatment. Surgical reduction of hyperplastic tissue by a periodontist (gingivectomy and gingivoplasty) may occasionally be required. **Periodontitis** Chronic periodontitis Chronic periodontitis (inflammation of the gingiva and periodontal membrane) may be a sequel of chronic gingivitis, usually because of accumulation of plaque and calculus. The gingiva detaches from the tooth, the periodontal membrane and alveolar bone are damaged, and an abnormal gap (pocket) develops between the tooth and gum. The tooth may slowly loosen and eventually be lost.

**Diagnosis**—Chronic periodontitis (pyorrhoea) is typically seen in adults. It is painless but may be associated with bleeding, halitosis, and a foul taste. Debris and pus may be expressed from the pockets, and there may be increasing tooth mobility. Periodontitis cannot be diagnosed by inspection alone, however, and requires specific diagnostic tests (periodontal probing and, sometimes, radiographs).

Chronic marginal gingivitis showing erythematous oedematous appearance

Gingivitis with hyperplasia

x Good oral hygiene is essential both in preventing and treating periodontal disease x

Antimicrobial drugs have no place in treating chronic gingivitis

Periodontitis with damage to supporting tissues including bone

Clinical review

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**Management**—Improvement in oral hygiene is necessary, but tooth brushing and mouthwashes have effect only above and slightly below the gum level. They are therefore ineffective in treating periodontitis, as plaque continues to accumulate below the gum line within periodontal pockets. Scaling and polishing and sometimes curettage are also required. Surgical removal of the pocket wall and diseased tissue may be needed to facilitate future cleansing, or attempts to regenerate lost periodontal tissue (such as guided tissue regeneration) may be indicated. Professional attention is therefore required. Although periodontal disease has a bacterial component, systemic antimicrobial drugs have no place in routine treatment, but topical treatment with antimicrobials within the periodontal pockets may be useful.

Periodontitis in young patients or which is rapidly advancing If periodontitis is seen in children or young adults or is rapidly advancing, systemic factors should be excluded.

**Gingival bleeding** Bleeding from the gingival margins is common and usually a consequence of gingivitis. It may be more obvious in women taking oral contraceptives and during the second and third trimesters of pregnancy. However, it may be a sign of platelet or vascular disorders and is common in leukaemia and HIV infection.

**Gingival ulcers** Gingival ulcers are often of infectious aetiology. Herpes simplex virus stomatitis is common in childhood but is increasingly seen in adults. There is a diffuse, purple, "boggy" gingivitis, especially anteriorly, with multiple vesicles scattered across the oral mucosa and gingiva. This is followed by ulcers. Diagnosis is usually clinical. The infection resolves spontaneously in 7-14 days, during which antipyretic analgesics such as paracetamol and adequate hydration are helpful. Antiviral drugs should be used in severe stomatitis and with immunocompromised patients, who may otherwise suffer severe infection. For those who can safely use it, an aqueous chlorhexidine mouthwash helps to maintain oral hygiene. **Acute necrotising ulcerative gingivitis**—Also known as Vincent's disease and trenchmouth, this affects mainly adults and causes painful ulceration of the gums between the teeth (interdental papillae), a pronounced tendency to gingival bleeding, and halitosis. Anaerobic fusiform bacteria and spirochaetes are implicated, and predisposing factors include poor oral hygiene, smoking, malnutrition, and immune defects including HIV and other viral infections and leukaemias. Management includes oral debridement and instruction on oral hygiene, peroxide or perborate mouthwashes, and metronidazole 200mg three times daily for three days. Other causes—Gingival ulcers may also be due to aphthae, self injury in psychologically disturbed or mentally challenged patients, malignant neoplasms, drugs, dermatoses, or systemic disease (haematological, mucocutaneous, gastrointestinal, or chronic infections such as tuberculosis, syphilis, mycoses, herpesviruses, and HIV).

x Good oral hygiene is essential in treating periodontitis + Periodontitis requires professional care, usually scaling and polishing and possibly periodontal surgery x Periodontitis that is unresponsive to such care, advances rapidly, or appears at an early age may have a systemic background

Causes of gingival bleeding Local x Gingivitis Chronic Acute necrotising x Periodontitis x Rarely, telangiectasia or angioma

Systemic x Thrombocytopenia Leukaemia HIV infection x Clotting defects Drugs such as anticoagulants

Herpetic stomatitis, with ulcerations on gingivae (top) and elsewhere in the mouth (bottom)

x Gingival ulceration in herpetic stomatitis is common and is associated with mouth ulcers elsewhere and fever x Gingival ulcers with bleeding and halitosis suggest a diagnosis of necrotising gingivitis

Necrotising (ulcerative) gingivitis

Clinical review

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**Gingival swelling** Widespread gingival swelling can be a feature of chronic gingivitis and may be caused by drugs, pregnancy, and systemic diseases. **Drug induced gingival swelling**—Drugs implicated include phenytoin, cyclosporin, and calcium channel blockers. Gingival swelling is usually worse if there is accumulation of plaque and calculus and the patient is receiving high drug doses. Improved oral hygiene and excision of enlarged tissue may be indicated. **Pregnancy gingivitis**—This usually develops around the

second month and reaches a peak in the eighth month. An exaggerated inflammatory reaction to plaque in pregnancy predisposes to gingivitis. Oral hygiene should be improved, particularly in view of the current concern that gingivitis may affect fetal birth weight. Pregnancy gingivitis tends to resolve on parturition. Hereditary gingival fibromatosis—This rare autosomal dominant condition may be associated with hirsutism, but most patients are otherwise perfectly healthy, although there are rare associations with systemic syndromes. Surgical reduction of the gingiva may be indicated. Gingival lumps Erupting teeth, particularly mandibular third molars, may be associated with swelling and tenderness of the overlying soft tissue flap (operculum), especially if this is traumatised by a tooth in the opposing dental arch. This condition, termed pericoronitis, is best treated by cleaning the area and having a dentist grind or remove the opposing tooth if it is causing trauma. If the patient is in severe pain or is feverish antimicrobials such as metronidazole 200mg thrice daily for up to five days may be indicated. Pregnancy epulis—Pregnancy may cause a localised swelling (epulis) of the gingival papillae, which may bleed or ulcerate. Occasionally, a large epulis requires surgical removal. Fibroepithelial polyp—Also called a fibrous lump and fibrous epulis, this is benign in nature. It may need to be removed. Malignant causes of gingival lumps include carcinoma, Kaposi's sarcoma, and lymphoma.

Red gingival lesions The most common cause of redness is gingivitis, in which the erythema is usually restricted to the gingival margins and interdental papillae (see above). Red lesions may also be due to desquamative gingivitis, erythroplasia, haemangiomas, orofacial granulomatosis, Crohn's disease, sarcoidosis, Wegener's granulomatosis, and neoplasms such as carcinoma and Kaposi's sarcoma.

Desquamative gingivitis Widespread erythema, particularly if associated with soreness, is usually caused by desquamative gingivitis. This is fairly common, is seen almost exclusively in women over middle age, and is usually a manifestation of lichen planus or mucous membrane pemphigoid. Its main features include persistent gingival soreness that is worse on eating and red gingivae. Diagnosis is usually obvious from the history and clinical features, but biopsy and immunostaining may be needed to establish the precise cause. Causes of gingival swelling Local causes x Chronic gingivitis causing Gingival abscesses Fibrous epulis x Hyperplastic gingivitis due to mouth breathing causing Exostoses Cysts Pyogenic granuloma Neoplasms

Systemic causes x Hereditary gingival fibromatosis and related disorders x Drugs (phenytoin, cyclosporin, calcium channel blockers) x Pregnancy x Sarcoidosis x Crohn's disease x Leukaemia x Wegener's granulomatosis x Rarely, amyloidosis, scurvy, midline lethal granuloma, mucopolysaccharidoses, mucopolysaccharidoses

Cyclosporin induced gingival swelling

Pregnancy gingivitis

Pregnancy epulis (pyogenic granuloma)

Desquamative gingivitis; usually a sign of pemphigoid (as here) or lichen planus

x Gingival swelling may occur in pregnancy and typically resolves at parturition x Gingival swelling may be drug induced—by phenytoin, cyclosporin, or calcium channel blockers

Clinical review

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features, but biopsy and immunostaining may be needed to establish the precise cause.

Management is based on treating the underlying condition and, if there are extraoral lesions, systemic treatment, usually with corticosteroids. Desquamative gingivitis can also

often be improved with better oral hygiene and topical corticosteroids such as fluocinonide cream used by a dentist in a plastic splint over the teeth and gums.

**Gingival pigmentation** This is usually a normal condition mainly seen in certain races (such as black people). Other causes include particles of dental amalgam embedded in the soft tissues, Addison's disease, Kaposi's sarcoma, drugs such as minocycline, melanotic macules and naevi, and melanoma.

**Halitosis** Oral malodour (foetor oris) predominantly originates from the tongue coating, gingival crevice, and periodontal pockets. Plaque organisms—especially *Porphyromonas gingivalis*, fusobacteria, and other anaerobes—cause putrefaction, resulting in release of volatile chemicals, particularly sulphide compounds (including hydrogen sulphide, methylmercaptan, dimethyl sulphide, and dimethyl disulphide). Some oral malodour is common in healthy individuals, particularly after sleep (morning breath). People who refrain from oral hygiene soon develop malodour, but this is worse with any form of sepsis of the aerodigestive tract such as gingivitis, periodontitis, dental abscess, dry socket, sinusitis, tonsillitis, nasal foreign bodies, and tumours. Many foods and drinks can cause malodour, especially garlic, onions, curries, the fruit durian, etc. Smoking and drugs—including alcohol, isosorbide dinitrate, and disulphiram—may also be implicated. Rare causes include diabetic ketoacidosis, renal or hepatic dysfunction, and psychiatric disease, as in delusional halitosis or as a feature in schizophrenia.

**Management** Management requires establishing the presence of true halitosis and assessing its severity with a portable sulphide monitor (halitometer). Dietary, infective, and systemic causes must be excluded. A full assessment of oral health is always indicated. The most reliably effective management is x Improving oral hygiene x Eating regularly x Avoiding odiferous foods, drugs, and other substances x Chewing sugar-free gum regularly x Using one of the many oral deodorants available over the counter x Using an antibacterial mouthwash or one such as Retardex or Dentyl x In severe or recalcitrant cases, using metronidazole 200mg thrice daily for seven days. John Coventry is senior lecturer in periodontology, Gareth Griffiths is senior lecturer in periodontology, Crispian Scully is dean, and Maurizio Tonetti is professor of periodontology at the Eastman Dental Institute for Oral Health Care Sciences, University College London, University of London ([www.eastman.ucl.ac.uk](http://www.eastman.ucl.ac.uk)). The ABC of Oral Health is edited by Crispian Scully and will be published as a book in autumn 2000. Crispian Scully is grateful for the advice of Rosemary Toy, general practitioner, Rickmansworth, Hertfordshire.

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**Mouthwashes with antimicrobial activity** Chlorhexidine gluconate x 0.1-0.2% aqueous mouthwash, rinse for 1 minute twice daily x Has measurable antiplaque activity x May stain teeth superficially if patient drinks tea, coffee, or red wine Povidone iodide x 1% mouthwash used 2-4 times daily for up to 14 days x Contraindicated in iodine sensitivity, pregnancy, thyroid disorders, or those taking lithium Cetylpyridinium chloride x 0.05% mouthwash used twice daily Hexetidine x 0.1% mouthwash used twice daily

Potential effects of tooth-brushing on human dentin wear following exposure to acidic soft drinks

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Keywords. Abrasion and erosion, AFM and SEM, dentin, soft drink, tooth-brushing.

**Summary** This study used scanning electron microscopy and atomic force microscopy to examine the short-term potential effects of brushing time and the start-time of tooth-brushing after demineralization on primary dentin wear in vitro. Thirty-six noncarious primary central incisors were assigned to 12 experimental groups. Exposure to cola drinks was used to initiate the demineralization process. Three brushing times (5, 15 and 30 s) and four start-times of brushing (0, 30, 60 and 120 min) after an erosive attack were used for the abrasion process. Tooth-brushing the softened dentin surface led to increases in the open tubular fraction and microstructural changes on the dentin surface. Brushing immediately after exposure to cola resulted in the greatest irreversible dentin loss, whereas brushing 60 or 120 min after pretreatment resulted in the least irreversible dentin loss. However, brushing time had no effect on their irreversible loss of dentin wear. Based on these experimental results, tooth-brushing should be performed at least 60 min after consuming a cola drink to achieve the desired tooth cleaning and avoid the introduction of surface lesions on dentin.

**Introduction** Increases in the consumption of dietary acids lead to increases in the occurrence of pathological tooth wear, usually defined as dental erosion (Hara et al., 2003). Dental erosion is recognized

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as an important cause of hard dental tissue loss. The chemical process of acidic dissolution leads to an irreversible loss of the outermost enamel and dentin layers and partial softening of the tooth surface in the absence of microorganisms (Attin et al., 2004; Hemingway et al., 2006; Choi et al., 2010a). Because the softened tooth surface is highly susceptible to abrasive wear, brushing can easily remove the superficially softened dental hard tissue. This increased susceptibility to abrasion is influenced not only by tooth brushes and toothpaste but also by such mild challenges as tooth-brushing without toothpaste (Eisenburger et al., 2003; Gregg et al., 2004). Erosive and abrasive processes are commonly observed in the mouth (Hara et al., 2003). Both clinical and experimental findings have shown that individual wear mechanisms rarely act alone but interact with each other. The most important interaction is increased susceptibility to abrasion caused by demineralization of dental hard tissue. In Korea, people tend to believe that tooth-brushing immediately after the consumption of food or acidic beverages is the best way to prevent dental lesions or achieving the desired tooth surface cleaning. However, this habit leads to increased tooth wear (Davis and Winter, 1980). Edward et al. (1998) showed that waiting to

brush teeth at least 1 h after an erosive attack can reduce tooth surface loss caused by tooth-brushing abrasion. This delay allows for rehardening of the softened tooth surface, a process mainly supported by saliva. Tooth structures, which constantly undergo demineralization and remineralization processes, consist of enamel, dentin and pulp (Choi et al., 2010a, b). Dentin consists of 50 vol% carbonated hydroxyapatite, 30 vol% type I collagen and 20 vol% oral fluid. Dentin also contains tubules that represent the tracks taken by odontoblastic cells from the dentinoenamel junction to the pulp. The tubules converge on the pulp and therefore tubule density and orientation vary

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from location to location. The number of dentinal tubules (DTs) per unit surface area is lowest at the dentinoenamel junction and highest at the dentin–pulp junction. Because dentin wears away more quickly than enamel, the brushing on dentin that is exposed by the removal of brackets or physical impact promotes surface abrasion and leads to tooth sensitivity including dental caries and periodontitis (Jones, 2001; Marshall et al., 1997; Perdigão, 2010). Data from clinical and in vitro studies (Bartlett and Smith, 2000; Hunter et al., 2002; Addy and Hunter, 2003) have shown that toothpaste is a major cause of abrasion and that it affects dentin much more than enamel. Absi et al. (1992) reported that brushing without toothpaste has no effect on enamel and clinically minimum effects on dentin. Most studies investigating the relationship between erosion and acidic soft drinks or abrasion and brushing are limited to enamel. A number of studies have demonstrated an interaction between dental abrasion and demineralization of dentin and reported that acid-softened dentin is vulnerable to tooth-brushing abrasion, both in situ (Hara et al., 2003; Attin et al., 2004) and in vitro (Davis and Winter, 1980; Attin et al., 2001; Ponduri et al., 2005; Vieira et al., 2006; Pinto et al., 2010; Zandim et al., 2010). Ponduri et al. (2005) showed that fluoride toothpaste could provide protection against dentin wear. Pinto et al. (2010) showed that tooth brushing could decrease the permeability of root dentin after exposure to acidic soft drinks. However, Attin et al. (2001) demonstrated that remineralization of eroded dentin does not improve susceptibility to dentin wear. Similarly, Hara et al. (2003) showed that exposure to saliva for 60 min has no protective effect on dentin wear. Attin et al. (2004) suggest that waiting to brush for at least 30 min after exposure to acidic drinks will help protect dentin wear. It is recommended in Korea to brush for 3 min immediately after a meal (Korean Dental Association, 2012). Therefore, the effects of brushing on irreversible dentin wear are controversial and strong evidence on the effects of brushing remains to be discovered. To the best of our knowledge, there are no reports on the potential effects of brushing time and the start-time of brushing on dentin wear after consumption of acidic soft drinks. Various assessment methods have been applied to evaluate the loss of dental hard tissue in enamel or dentine induced by erosive challenges, particularly scanning electron microscopy (SEM) and atomic force microscopy (AFM). Although environmental SEM allows for examination of samples without additional process in wet conditions, SEM and environmental SEM assessments do not provide detailed information about surface alternations of eroded enamel and dentine surfaces (Choi et al., 2012). In contrast, AFM provides the three-dimensional (3D) configuration with quantitative information regarding of the surface morphology with

minimal sample preparation. Therefore, this study used AFM and SEM to examine the potential effects of brushing on dentin surfaces demineralized by cola drinks in vitro and to evaluate the proper start-time of brushing after the consumption of acidic soft drinks.

#### Materials and methods

**Preparation of dentin slabs** The 360 primary central incisors used in this study were surgically extracted without carious lesions or dental fluorosis.

All primary teeth were disinfected using a solution containing hydrogen peroxide, washed with a physiological phosphate buffered saline (PBS) solution with pH 7.4 and then immersed in PBS solution at room temperature for a maximum of

2 months. The teeth were then re-washed in PBS and embedded in epoxy resin. Dentin slabs were prepared by removing the vestibular enamel layer from the cervical portion of the crown and cutting a section 2 mm long, 1 mm wide and 3 mm thick using a low speed diamond saw (IsoMet, Buehler Ltd., Lake Bluff, IL, USA). The specimens were polished sequentially with 320 grit paper for 90 s, 400 grit for 3 min and 600 grit for 3 min

under distilled water. Fine polishing was performed for 3 min using water-based suspensions of 6- and 1- $\mu$ m polycrystalline diamond particles (IsoMet, Buehler Ltd., Lake Bluff, IL, USA).

The polished dentin slabs were then rinsed with PBS.

**Demineralization: exposure to cola drinks** Exposure to cola drinks (Coca-Cola Enterprises Ltd., Yangsan, Korea) was used for the demineralization process. Half of the area of each dentin slab was covered with nail varnish and this area was used as a reference to determine the etching depth caused by acidic soft drink. Each dentin slab was immersed in 50 mL of cola drink (pH 2.52) for 15 min (Chuenarrom and Benjakul, 2008; Hon'orio et al., 2010; Lodi et al., 2010; Shellis et al., 2010; Torres et al., 2010; Soares et al., 2011) and rinsed with PBS. Nail polish remover was applied over the nail varnish covering the reference area until clean.

**Abrasion: tooth-brushing** Tooth-brushing with an electric toothbrush (Braun Oral-B Triumph IQ 5000, Procter & Gamble, OH, USA) was used for the abrasion process. There were 12 experimental groups (Table 1, n = 30 per group) established according to three brushing times and four start-times of tooth-brushing after an erosive attack. The brushing times were performed with an electric toothbrush filled with 2 gm in 1.5 mL water slurry fluoride toothpaste (Dental 2080 Original Toothpaste, 150g, Aekyung, Seoul, Korea) for 5, 15 and 30 s at a frequency of four strokes per second and a load of 4 N. This brushing time was calculated by dividing the conventional recommendation time in Korea (3 min) or in Europe (2 min) by the number of teeth, approximately 6.42 or 4.28 tooth<sup>-1</sup>. The start-times of

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Table 1. Description of the 12 experimental trials (n = 30 per group) according to brushing time and the start-time of tooth-brushing after an erosive attack with cola soft drinks.

Start-time of tooth-brushing after demineralization (min)

Brushing time (s) Immediately 30 60 120

5 Group1 Group2 Group3 Group4 15 Group5 Group6 Group7 Group8 20 Group9 Group10 Group11 Group12

Table 2. Description of the five diagnostic parameters proposed in this study.

Analytic parameter Description

rdDT Change in the dentinal tubule diameter between the exposed and reference areas.

Eq.(1)

rhDT Change in the dentinal tubule depth (height) between the exposed and reference areas.

Eq.(2)

rnDT Change in the number of dentinal tubules between the exposed and reference areas.

Eq.(3)

rRa Change in the line roughness average between the exposed and reference areas.

Eq.(4)

rRq Change in the line root mean square (RMS) roughness between the exposed and reference areas.

Eq.(5)

tooth-brushing were immediately, 30, 60 and 120 min after demineralization with cola drinks. After brushing, each tooth was rinsed with PBS and dried with an air syringe.

Wear assessment: AFM examination Non-contact mode AFM images were obtained using a NANOstation II (Surface Imaging Systems, Herzogenrath, Germany) equipped with a  $92.5 \times 92.5 \times 6 \mu\text{m}^3$  XYZ scanner and a Zeiss optical microscope (Epiplan 100 $\times$  and 500 $\times$ ). The AFM was placed on an active vibration isolation table (TS-150, S.I.S., Herzogenrath, Germany) inside a passive vibration isolation table (Pucotech, Seoul, Republic of Korea) to eliminate external noise. All AFM dentinal images were scanned in air with 35% relative humidity at a resolution of  $256 \times 256$  pixels, a scan speed of 0.8 lines/s and image sizes of  $50 \times 50 \mu\text{m}^2$  using a silicon cantilever with an integral pyramidal shaped tip (SICONG, Santa Clara, CA, USA). AFM dentinal images consisting of three from each dentin slab were obtained from this study. Five changes in dentin surface morphology, Table 2 and Eqs. (1)–(5), were measured by three observers blinded to experimental group assignment, using a Scanning Probe Image Processor version 4.8 (SPIP, Image Metrology, Lyngby, Denmark).

rdDT = DT diameter in A area / DT diameter in B area, (1)

rhDT =

DT depth in A area / DT depth in B area

, (2)

rnDT =

Number of DTs in A area / Number of DTs in B area

, (3)

rRa =

Line roughness average in A area / Line roughness average in B area

, (4)

rRq =

Line RMS roughness in A area / Line RMS roughness in B area

. (5)

where A indicates the areas of exposed dentin in vitro whereas B indicates the areas which are protected with nail varnish during the clinical trials. After AFM imaging, the specimens were used again for SEM.

**Wear assessment:** SEM examination SEM (Hitachi S-4700, Hitachi Co. Ltd., Tokyo, Japan) was used to evaluate changes in the morphology of dentin surfaces with demineralization and abrasion. All dentin slabs were immobilized on brass stubs with double-sided tape and vacuum coated with a layer of carbon followed by gold. The surfaces of each block were then examined at a beam voltage of 10 kV.

**Statistical analysis** The quantitative data are expressed as the mean  $\pm$  standard deviation (SD). The Kolmogorov–Smirnov test was used to confirm that quantitative data were normally distributed. One-way analysis of variance (ANOVA) was performed to compare morphological differences among the groups, including the rate of change for three DT features (rdDT, rhDT and rnDT) and two line roughness features (rRa and rRq) between the exposed and reference areas. Additional post hoc comparisons were performed using a Student–Newman–Keuls test where appropriate. *p*-Values  $< 0.05$  were considered significant.

**Results** Figure 1 shows representative SEM images of dentin surfaces brushed for 15 s (groups 5–8) in sequence from erosive to abrasive. Areas of exposed dentin *in vitro* are indicated by A whereas B indicates the areas which are protected with nail varnish during the clinical trials. In most cases, the exposed areas appeared brighter and showed widened DTs compared to the reference areas. The rdDTs were inversely proportional to the start-time of tooth-brushing after demineralization with cola drink ( $p < 0.001$ ). The exposed areas of group 5, brushed immediately after erosive attacks, showed an increase in rdDT of  $2.28 \pm 0.19$  times compared to the reference areas. Unlike group 5, the exposed areas for groups 7 and 8, brushed 60 and 120 min, respectively, after erosive attacks, had rdDT similar

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Fig. 1. Representative SEM images (500 $\times$  and insets 2000 $\times$ ) of dentin surfaces brushed for 15 s (a) immediately: group 5, (b) 30 min: group 6, (c) 60 min: group 7 and (d) 120 min: group 8 after demineralization with cola drinks. In each panel, the areas marked by A were preexposed to a 50 mL cola soft drink and brushed with an automatic brushing machine, whereas those marked by B were covered with nail varnish during exposure to cola drinks.

to the reference areas with increases of only  $1.06 \pm 0.07$  and  $1.02 \pm 0.06$  times, respectively.

Figure 2 shows representative AFM topography images, line profiles and AFM 3D images of dentin surfaces brushed for 5 s (groups 1–4) in sequence. The exposed and reference areas are indicated by A and B, respectively. The exposed areas of all groups clearly showed an opening of DTs and widened tubular lumens, which were caused by acid attack and tooth brushing. A substantial amount of debris and deposits were also observed. Line profiles and AFM 3D images showed the elaborate nanostructures of dentin surfaces eroded and abraded by immersion in a 50 mL cola drink and brushing with an automatic brushing machine. In most cases, the exposed areas appeared lower than the reference areas. The rdDT and rhDT in the exposed areas were wider and deeper than those in the reference areas. These changes in tubular morphology, between exposed and reference areas, decreased as the start time of brushing after demineralization increased ( $p < 0.001$ ).

Figure 3 shows three DT features, including rdDT (Figs 3a and b), rhDT (Fig. 3c) and rnDT (Fig. 3d) and two line roughness features, including rRa (Fig. 3e) and rRq (Fig. 3f), for each group. The peritubular dentin was excluded from the calculation of tubular diameter and depth. The group that was brushed immediately after demineralization showed irreversible dentin wear that was significantly greater ( $p < 0.001$ ) compared to the groups that were brushed 60 or 120 min after demineralization. The group that brushed 60 min after an erosive attack began showing a slight change in tubular morphology between two areas. This change was maintained until the next group, which were brushed 120 min after an erosive attack. All features showed the same progressive pattern except for rnDT. Statistical information regarding the diagnostic parameters is summarized in Tables 3 and 4.

**Discussion** We examined the potential effects of brushing time and start-time of tooth-brushing after exposure to cola drinks on irreversible dentin wear in vitro. Immersion in cola drinks and tooth-brushing were used as the erosive and abrasive procedures, respectively. Tooth-brushing on dentin surfaces preeroded by exposure to cola drinks led to morphological changes including roughness and increases in the open tubular fraction and tubular diameter and depth.

Tooth brushing immediately after an erosive attack with cola drinks showed the most irreversible dentin wear, whereas 60 or 120 min after an erosive attack showed the least irreversible dentin wear ( $p < 0.001$ ). Abrasion from tooth-brushing with preerosion was inversely proportional to the start-time of brushing after an erosive attack. Interestingly, tooth-brushing 60 or 120 min after demineralization showed no significant

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Fig. 2.

Representative AFM topographic images, line profile and AFM 3D images of dentin surfaces brushed for 5 s (a) immediately: group 1, (b) 30 min: group 2, (c) 60 min: group 3 and (d) 120 min: group 4 after an erosive attack with cola soft drinks. In each panel, the areas marked by A were preexposed to a 50 mL cola drink and brushed with an automatic brushing machine, whereas those marked by B were recovered with nail varnish during exposure to cola drinks.

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Fig. 3. Three DT parameters including (a, b) rdDT, (c) rhDT and (d) rnDT and two line roughness parameters including (e) rRa and (f) rRq are shown for each group. The groups that brushed immediately after an erosive attack showed irreversible dentin loss that was significantly higher ( $p < 0.001$ ) compared to the groups that brushed 60 or 120 min after an erosive attack. All diagnostic parameters showed the same progressive pattern. The rdDT (SEM) indicates rdDT finding obtained from SEM assessment and other parameters indicate findings obtained from AFM assessments.

difference in irreversible dentin wear. The brushing time had no effect on the erosive and/or abrasive potential of dentin. An increase in the consumption of

acidic products has resulted in an increase in the incidence of dental lesions including dental caries or dental erosion. Dental caries is a process of demineralization of hard dental tissues. It is

caused by acids excreted by oral bacteria (a byproduct of carbohydrate metabolism) that diffuse into the enamel and then into the dentin, dissolving mineral and destroying the structural matrix (Bertassoni et al., 2010). On the other hand, unlike dental caries, dental erosion is the progressive and irreversible loss of tooth surface by a chemical process of C

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Table 3. Normality test of diagnostic parameters (Kolmogorov–Smirnov test).

Treatment After erosive SEM AFM Group Brushing time (s) attack (min) rdDT rdDT rhDT rRa rRq rnDT

1 5 Immediately =0.309 =0.735 =0.959 =0.885 =0.372 =0.385 2 5 30 =0.654 =0.957 =0.888 =0.326 =0.846 =0.674 3 5 60 =0.304 =0.059 =0.667 =0.917 =0.339 =0.589 4 5 120 =0.346 =0.380 =0.465 =0.083 =0.058 =0.321 5 15 Immediately =0.664 =0.308 =0.409 =0.932 =0.724 =0.563 6 15 30 =0.158 =0.573 =0.153 =0.471 =0.523 =0.757 7 15 60 =0.319 =0.236 =0.949 =0.793 =0.689 =0.710 8 15 120 =0.319 =0.056 =0.947 =0.521 =0.304 =0.504 9 30 Immediately =0.610 =0.809 =0.940 =0.810 =0.699 =0.280 10 30 30 =0.576 =0.769 =0.619 =0.368 =0.227 =0.172 11 30 60 =0.570 =0.078 =0.290 =0.435 =0.209 =0.379 12 30 120 =0.308 =0.082 =0.240 =0.990 =0.967 =0.501

acidic dissolution without the involvement of bacteria. It has recently been recognized as an important cause of tooth loss (National Institutes of Health U.S., 2001; Hemingway et al., 2006; Choi et al., 2010a). The acid causes decalcification, creating irregularities on the tooth surface (Attin et al., 2004; Hemingway et al., 2006; Brown et al., 2007; Choi et al., 2010a). Many studies investigating the effects of

remineralization solution on tooth surfaces have concentrated

on enamel and not dentin (Brown et al., 2007). Bertassoni et al. (2010) examined the morphological and mechanical changes on dentin surfaces in response to treatment with a remineralization solution and showed that continuous delivery of calcium and phosphate leads to remineralization and mechanical recovery of dentin. Featherstone and Lussi (2006) stated that the dental erosion by acidic beverages is directly affected by the presence in this beverage of both

Table 4. ANOVA and post hoc comparisons of progressive changes on dentin surfaces according to brushing time and the start-time of tooth-brushing after an erosive attack\*.

Treatment SEM Brushing After erosive AFM Group time (s) attack (min) rdDT rdDT rhDT rRa rRq rnDT 1 5 Immediately 2.09±0.11a 1.91±0.03a 3.07±0.09a 2.10±0.05a 2.06±0.07a 1.03±0.03 2 5 30 1.52±0.18b 1.56±0.08b 2.60±0.19b 1.87±0.01a 1.66±0.01b 0.98±0.04 3 5 60 1.06±0.05c 1.23±0.22c 1.42±0.19c 1.33±0.01b 1.35±0.01c 0.98±0.05 4 5 120 1.07±0.02c 1.20±0.19c 1.20±0.19c 1.11±0.01b 1.14±0.01d 1.08±0.09 p-Value (ANOVA) <0.001 <0.001 <0.001 <0.001 <0.001 NS† 5 15 Immediately 2.28±0.19a 2.14±0.16a 3.47±0.21a 2.59±0.01a 2.54±0.01a 1.01±0.02 6 15 30 2.15±0.11b 2.10±0.05a 3.15±0.18a 2.17±0.00b 2.14±0.01b 1.18±0.12 7 15 60 1.06±0.07c 1.33±0.16b 1.51±0.15b 1.17±0.01c 1.10±0.01c 1.05±0.03 8 15 120 1.02±0.06c 1.27±0.11b 1.29±0.15b 0.96±0.02c 1.15±0.02c 0.99±0.05 p-Value (ANOVA) <0.001 <0.001 <0.001 <0.001 <0.001 NS† 9 30 Immediately 2.50±0.24a 2.24±0.24a 3.39±0.38a 3.39±0.03a 3.77±0.03a 1.15±0.13 10 30 30 2.08±0.24b 2.25±0.07a

2.72±0.09b 2.25±0.04b 2.33±0.05b 1.23±0.14 11 30 60 1.02±0.15c 1.34±0.19b 1.37±0.15c  
 0.94±0.01c 0.84±0.01c 1.02±0.09 12 30 120 1.08±0.11c 1.13±0.01b 1.21±0.16c 1.17±0.01c  
 1.17±0.00c 1.09±0.08 p-Value (ANOVA) <0.001 <0.001 <0.001 <0.001 <0.001 NS†

\*Labelswithdifferentlettersindicateasignificantdifference(Student–Newman–Keulstest,p < 0.05)afterANOVA. †NS=notsignificant.

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phosphoric and citric acids, which provide mechanisms of dissolution by removal of carbonate or phosphate ions by hydrogen ions and removal of calcium by binding with chelating agents. Wongkhantee et al. (2006) examined the Vicker microhardness change ( VHN) of dentin surfaces immersed in a combination of artificial saliva and one of four different acidic drinks including cola (pH 2.74), orange juice (pH 3.75), sports drink (pH 3.78) and drinking yogurt (pH 3.83) for 5 s, a total of 10 times. The cola drink (3.27

VHN)showedthemostsignificantdecreaseofdentinsurface hardnesscomparedtoorangejuice(0.81 VHN),sportsdrink (0.47 VHN)ordrinkingyogurt(0.02 VHN).Inaddition,apreliminary study by Cheong et al. (2010) reported that cola drinks (mean surface roughness = 1861.38 nm) have the greatesterosivepotentialcomparedtoPowerade(745.07nm), orange juice (362.13 nm) or milk (159.34 nm) for the same treatmenttimeof60min.Therefore,inthisstudy,coladrinks were selected for the demineralization process. Indeed, the erosive potential of cola drinks on dentin in this study was similar to that reported previously by Wiegand and Attin (2007) and Jensdottir et al. (2006). Shellis et al. (2010) reported the effects of pH and acid concentration on the dissolution rate of dentin in three acid solutions (pH 2.45, pH3.2 andpH 3.9)anddemonstrated aprogressive decrease in dissolution rate with respect to time on dentin. This result is consistent with the present study, in which the abrasive and erosive potential decreased with increasing start-time of tooth-brushingafteranerosiveattackwithacoladrink. Dentin (3 on Mohs hardness scale) is softer than enamel (5 on Mohs hardness scale) and wears away more rapidly than enamel. Therefore, dentin exposure causes symptoms of sensitive teeth associated with dental caries, periodontitis, tooth demineralization, bulimia nervosa, dental abscesses, gumdisordersandsoon.Dentinconsistsofdistinctstructures calledDTs,whereasenamelhasaspecificstructureconsisting ofaverticalarrayofmineralprisms.DTsrepresentthetracks takenbyodontoblasticcellsfromthedentinoenameljunction to the pulp. The tubules converge on the pulp and therefore tubuledensityandorientationdifferaccordingtothelocation. The number of DTs per unit surface area is lowest at the dentinoenamel junction and highest at the dentin and pulp junction. Therefore, in this study, three dentinal features, diameter, depth and number of DTs and two line roughness features (mean roughness and root mean square roughness) were selected to determine the start-time of brushing after an erosive attack on dentin. It was hypothesized that toothbrushingafteranerosiveattackwouldaltertubulardiameter (hypothesis 1) and the number of tubules (hypothesis 2: abrasive and erosive processes expose invisible tubules). The presence of tubular deposits affects the demineralization response (Prati et al., 2003) and acidic soft drinks lead to increased dentinal permeability

by removing and dissolving the smear layer and smear plugs (Vieira et al., 2006). Therefore, tubular diameter is easily widened by abrasion from tooth-brushing. This study showed that demineralization caused by cola drinks and abrasion from brushing leads to a significant increase in tubular diameter. Furthermore, tubular structure and the density of dentin depend on location. In this study, target areas of dentin surfaces were randomly selected. Therefore, the number, size and density of DTs differed from each other according to group. However, because comparisons were made at the same location for each group, these differences among groups were not discussed. Results from the comparisons performed among the groups showed that the  $r_nDT$ , the change in the size ratio of DTs between the exposed and reference areas, did not differ between the exposed and reference areas with respect to the start-time of brushing after demineralization. Therefore, these findings support hypothesis 1 but not hypothesis 2. A number of studies have reported on the effects of tooth brushing on acid-softened dentin *in situ* and *in vitro* (Davis and Winter, 1980; Attin et al., 2001, 2004; Hara et al., 2003; Ponduri et al., 2005; Vieira et al., 2006; Pinto et al., 2010; Zandim et al., 2010). However, the potential effects of tooth-brushing on irreversible dentin wear are still controversial. It is well known that abrasion from tooth-brushing is influenced by the force (load), start-time, frequency and duration of brushing. Therefore, it is advisable to perform tooth-brushing three times a day with minimal force for 3 min immediately after having a meal. Heintze et al. (2010) showed that a 350 g load is comparable to the load used in clinical studies, whereas other studies have shown that a load of 200 g (Neme et al., 2002), 250 g (Teixeira et al., 2005), 350 g (Tanoue et al., 2000) or 500 g (Cho et al., 2002) is best. Therefore, in this study, tooth-brushing with a load of approximately 400 g and four brush strokes per second was performed during a single brushing episode. By following this brushing protocol we expected to accurately simulate clinical conditions. The results of this study showed that abrasion (tooth-brushing) immediately after demineralization (cola drink) showed the largest abrasive/erosive potential on dentin. This result is consistent with that reported by Vieira et al. (2006). If teeth are initially softened by an acid solution, they are more likely to be disrupted by mechanical factors, even tooth-brushing without toothpaste or friction caused by the tongue. This increased susceptibility decreases according to the time elapsed after demineralization. Attin et al. (2004) showed that irreversible dentin loss begins to decrease by waiting to brush 30 min after an erosive attack. The difference between the present study (at least 60 min) and the study by Attin (at least 30 min) is probably because of the action of saliva. In addition, this study showed no correlation between brushing time and irreversible dentin wear. Therefore, a conventional recommendation time of 2 min (in Europe) or 3 min (in Korea) is sufficient for brushing and a longer brushing time merely irritates the gingival tissues without achieving the desired tooth cleaning. In this study, dentin samples were sectioned by removing the vestibular C

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enamel layer from the cervical portion of the crown so that the location of an acid attack would be similar to that *in vivo*. However, there were some limitations to this study

including not investigating the action of saliva or different exposure times to cola drinks. Therefore, further studies will be needed to examine the effects of a range of factors on acid erosion, such as different types of acidic soft drinks, exposure time or addition of saliva. In conclusion, we evaluated the effects of start-time of tooth-brushing after the consumption of acidic soft drinks. The potential loss of dentin because of abrasion and/or erosion was inversely proportional to the start-time of toothbrushing after an erosive attack. Tooth-brushing 60 or 120 min after demineralization showed no significant difference on irreversible dentin wear. This suggests that, under our experimental conditions, tooth-brushing should be performed at least 60 min after the consumption of cola drinks to achieve less abrasion and/or erosion on dentin. Brushing time had no effect on dentin wear. Therefore, under experimental conditions, a 3 min brushing time after the consumption of cola drinks is sufficient for preventing dental lesions and a longer brushing time is not necessary. Finally, it is obvious that AFM provides an nanometric resolution of the 3D dentinal images and a more precise measure of dentinal abrasive and erosive potentials.

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## 12. What condition is NOT a part of Metabolic Syndrome:

- a. Large waistline
- b. High triglyceride
- c. High blood pressure
- d. High HDL-cholesterol
- e. All are conditions of metabolic syndrome

What is metabolic syndrome? Metabolic syndrome is the name for a group of risk factors for heart disease [<https://medlineplus.gov/heartdiseases.html>] , diabetes [<https://medlineplus.gov/diabetes.html>] , and other health problems. You can have just one risk factor, but people often have several of them together. When you have at least three of them, it is called metabolic syndrome. These risk factors include A large waistline, also called abdominal obesity or "having an apple shape." Too much fat around the stomach is a greater risk factor for heart disease than too much fat in other parts of the body. Having a high triglyceride [<https://medlineplus.gov/triglycerides.html>] level. Triglycerides are a type of fat found in the blood. Having a low HDL cholesterol level [<https://medlineplus.gov/hdlthegoodcholesterol.html>] . HDL is sometimes called the "good" cholesterol because it helps remove cholesterol from your arteries. Having high blood pressure [<https://medlineplus.gov/highbloodpressure.html>] . If your blood pressure stays high over time, it can damage your heart and lead to other health problems. Having a high fasting blood sugar [<https://medlineplus.gov/bloodsugar.html>] . Mildly high blood sugar may be an early sign of diabetes. The more factors you have, the higher your risk for heart disease, diabetes, and stroke [<https://medlineplus.gov/stroke.html>] is.

What causes metabolic syndrome? Metabolic syndrome has several causes that act together:

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COVID-19 is an emerging, rapidly evolving situation. Get the latest public health information from CDC: <https://www.coronavirus.gov> Get the latest research information from NIH: <https://www.nih.gov/coronavirus>

x

Metabolic Syndrome | MedlinePlus <https://medlineplus.gov/metabolicsyndrome.html#>  
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Overweight and obesity [<https://medlineplus.gov/obesity.html>] An inactive lifestyle [<https://medlineplus.gov/healthrisksofaninactivelifestyle.html>] Insulin resistance, a condition in which the body can't use insulin properly. Insulin is a hormone that helps move blood sugar into your cells to give them energy. Insulin resistance can lead to high blood sugar levels. Age - your risk goes up as get older Genetics - ethnicity and family history People who have metabolic syndrome often also have excessive blood clotting [<https://medlineplus.gov/bloodclots.html>] and inflammation throughout the body. Researchers don't know whether these conditions cause metabolic syndrome or worsen it. Who is at risk for metabolic syndrome? The most important risk factors for metabolic syndrome are Abdominal obesity (a large waistline) An inactive lifestyle Insulin resistance There are certain groups of people who have an increased risk of metabolic syndrome: Some racial and ethnic groups. Mexican Americans have the highest rate of metabolic syndrome, followed by whites and blacks. People who have diabetes People who have a sibling or parent who has diabetes Women with polycystic ovary syndrome

[<https://medlineplus.gov/polycysticovarysyndrome.html>] (PCOS) People who take medicines that cause weight gain or changes in blood pressure, blood cholesterol, and blood sugar levels What are the symptoms of metabolic syndrome? Most of the metabolic risk factors have no obvious signs or symptoms, except for a large waistline.

How is metabolic syndrome diagnosed? Your health care provider will diagnose metabolic syndrome based on the results of a physical exam and blood tests. You must have at least three of the risk factors to be diagnosed with metabolic syndrome: A large waistline, which means a waist measurement of 35 inches or more for women 40 inches or more for men A high triglyceride level, which is 150 mg/dL or higher A low HDL cholesterol level, which is Metabolic Syndrome | MedlinePlus <https://medlineplus.gov/metabolicsyndrome.html#> 2 of 6 4/20/20, 2:22 PM

Less than 50 mg/dL for women Less than 40 mg/dL for men High blood pressure, which is a reading of 130/85 mmHg or higher. A high fasting blood sugar, which is 100 mg/dL or higher What are the treatments for metabolic syndrome? The most important treatment for metabolic syndrome is a heart-healthy lifestyle, which includes A heart-healthy eating plan, which limits the amount of saturated and trans fats that you eat. It encourages you to choose a variety of nutritious foods, including fruits, vegetables, whole grains, and lean meats. Aiming for a healthy weight [<https://medlineplus.gov/weightcontrol.html>]

Managing stress [<https://medlineplus.gov/stress.html>] Getting regular physical activity [<https://medlineplus.gov/howmuchexercisedoineed.html>] Quitting smoking [<https://medlineplus.gov/quittingsmoking.html>] (or not starting if you don't already smoke) If making lifestyle changes is not enough, you may need to take medicines. For example, you may need medicines to lower cholesterol or blood pressure.

Can metabolic syndrome be prevented? The best way to prevent metabolic syndrome is through the heart-healthy lifestyle changes. NIH: National Heart, Lung, and Blood Institute Start Here Metabolic Syndrome [<https://familydoctor.org/condition/metabolic-syndrome/?adfree=true>] (American Academy of Family Physicians) Also in Spanish [<https://es.familydoctor.org/condicion/sindrome-metabolico/?adfree=true>] Metabolic Syndrome [<https://www.nhlbi.nih.gov/health-topics/metabolic-syndrome>] (National Heart, Lung, and Blood Institute) Symptoms Symptoms and Diagnosis of Metabolic Syndrome [<https://www.heart.org/en/health-topics/metabolic-syndrome/symptoms-and-diagnosis-of-metabolic-syndrome>] (American Heart Association) Related Issues Diabetes, Heart Disease, and Stroke [<https://www.niddk.nih.gov/health-information/diabetes/overview/preventing-problems/heart-disease-stroke>]

Metabolic Syndrome | MedlinePlus <https://medlineplus.gov/metabolicsyndrome.html#> 3 of 6 4/20/20, 2:22 PM

(National Institute of Diabetes and Digestive and Kidney Diseases) Also in Spanish [<https://www.niddk.nih.gov/health-information/informacion-de-la-salud/diabetes/informacion-general/prevenir-problemas/diabetes-enfermedad-corazon-ataques-cerebrales>] Insulin Resistance and Prediabetes [<https://www.niddk.nih.gov/health-information/diabetes/overview/what-is-diabetes/prediabetes-insulin-resistance>]

(National Institute of Diabetes and Digestive and Kidney Diseases) Also in Spanish [<https://www.niddk.nih.gov/health-information/informacion-de-la-salud/diabetes/informacion-general/que-es/resistencia-insulina-prediabetes>] Obesity [<https://www.hormone.org/diseases-and-conditions/obesity>] (Hormone Health Network) Also in Spanish [<https://www.hormone.org/pacientes-y-cuidadores/las-hormonas-y-la>

Also in Spanish [<https://www.hormone.org/pacientes-y-cuidadores/las-hormonas-y-la>

obesidad] Statistics and Research Metabolic Syndrome Prevalence by Race/Ethnicity and Sex in the United States, National Health and Nutrition Examination Survey, 1988-2012 [https://www.cdc.gov/pcd/issues /2017/16\_0287.htm] (Centers for Disease Control and Prevention) Clinical Trials ClinicalTrials.gov: Insulin Resistance [https://clinicaltrials.gov/search /open/condition=%22Insulin+Resistance%22] (National Institutes of Health) ClinicalTrials.gov: Metabolic Syndrome [https://clinicaltrials.gov/search /open/condition=%22Metabolic+Syndrome%22] (National Institutes of Health) Journal Articles References and abstracts from MEDLINE/PubMed (National Library of Medicine) Article: Sex-specific association of hyperuricemia with cardiometabolic abnormalities in a military cohort:... [https://www.ncbi.nlm.nih.gov/pubmed/32195957] Article: Improving prevention strategies for cardiometabolic disease. [https://www.ncbi.nlm.nih.gov/pubmed/32152584] Article: Prevalence of and associations between metabolic syndrome and the constitutions defined... [https://www.ncbi.nlm.nih.gov/pubmed/32049808] Metabolic Syndrome -- see more articles [https://www.ncbi.nlm.nih.gov /pubmed?term=metabolic+syndrome+x[majr]+AND+english[la]+AND+humans[mh]+AND + (patient+education+handout[pt]+OR+guideline[pt]+OR+review[pt]+OR+clinical+trial[pt]+ OR +jsubsetk[text]+OR+jsubsetaim[text]+OR+jsubsetn[text])+NOT+ (letter[pt]+OR+editorial[pt])+AND+%22last+1+Year%22[edat]] Find an Expert American Diabetes Association [https://www.diabetes.org] American Heart Association [https://www.heart.org/en/] National Institute of Diabetes and Digestive and Kidney Diseases [https://www.niddk.nih.gov]

### 13. Which food does NOT cause inflammation in the gut?

- a. Added sugar
- b. Wheat
- c. Omega-6 fatty acid
- d. Omega-3 fatty acid
- e. All are not inflammatory

6 Foods That Cause Inflammation Inflammation can be good or bad depending on the situation.

On one hand, it's your body's natural way of protecting itself when you're injured or sick. It can help your body defend itself from illness and stimulate healing.

On the other hand, chronic, sustained inflammation is linked to an increased risk of diseases like diabetes, heart disease, and obesity (1Trusted Source, 2Trusted Source, 3Trusted Source).

Interestingly, the foods you eat can significantly affect inflammation in your body.

Here are 6 foods that can cause inflammation. 1. Sugar and high-fructose corn syrup Table sugar (sucrose) and high fructose corn syrup (HFCS) are the two main types of added sugar in the Western diet.

Sugar is 50% glucose and 50% fructose, while high fructose corn syrup is about 45% glucose and 55% fructose.

One of the reasons that added sugars are harmful is that they can increase inflammation, which can lead to disease (4Trusted Source, 5Trusted Source, 6Trusted Source, 7Trusted Source, 8Trusted Source).

In one study, mice fed high sucrose diets developed breast cancer that spread to their lungs, partly due to the inflammatory response to sugar (6Trusted Source).

In another study, the anti-inflammatory effects of omega-3 fatty acids were impaired in mice fed a high sugar diet (7Trusted Source). What's more, in a randomized clinical trial in which people drank regular soda, diet soda, milk, or water, only those in the regular soda group had increased levels of uric acid, which drives inflammation and insulin resistance (8Trusted Source).

Sugar can also be harmful because it supplies excess amounts of fructose.

While the small amounts of fructose in fruits and vegetables are fine, consuming large amounts from added sugars is a bad idea. Eating a lot of fructose has been linked to obesity, insulin resistance, diabetes, fatty liver disease, cancer, and chronic kidney disease (9Trusted Source, 10Trusted Source, 11Trusted Source, 12Trusted Source, 13Trusted Source, 14Trusted Source, 15Trusted Source).

Also, researchers have noted that fructose causes inflammation within the endothelial cells that line your blood vessels, which is a risk factor for heart disease (16Trusted Source).

High fructose intake has likewise been shown to increase several inflammatory markers in mice and humans (10Trusted Source, 17Trusted Source, 18Trusted Source, 13Trusted Source, 19Trusted Source, 20Trusted Source).

Foods high in added sugar include candy, chocolate, soft drinks, cakes, cookies, doughnuts, sweet pastries, and certain cereals. SUMMARY Consuming a diet high in sugar and high fructose corn syrup drives inflammation that can lead to disease. It may also counteract the

anti-inflammatory effects of omega-3 fatty acids. 2. Artificial trans fats Artificial trans fats are likely the unhealthiest fats you can eat.

They're created by adding hydrogen to unsaturated fats, which are liquid, to give them the stability of a more solid fat.

On ingredient labels, trans fats are often listed as partially hydrogenated oils.

Most margarines contain trans fats, and they are often added to processed foods to extend shelf life.

Unlike the naturally occurring trans fats found in dairy and meat, artificial trans fats have been shown to cause inflammation and increase disease risk (21Trusted Source, 22Trusted Source, 23Trusted Source, 24Trusted Source, 25Trusted Source, 26Trusted Source, 27Trusted Source, 28Trusted Source, 29Trusted Source). In addition to lowering HDL (good) cholesterol, trans fats may impair the function of the endothelial cells lining your arteries, which is a risk factor for heart disease (26Trusted Source).

Consuming artificial trans fats is linked to high levels of inflammatory markers, such as C-reactive protein (CRP).

In fact, in one study, CRP levels were 78% higher among women who reported the highest trans fat intake (26Trusted Source). In a randomized controlled trial including older women with excess weight, hydrogenated soybean oil increased inflammation significantly more than palm and sunflower oils (27Trusted Source).

Studies in healthy men and men with elevated cholesterol levels have revealed similar increases in inflammatory markers in response to trans fats (28Trusted Source, 29Trusted Source).

Foods high in trans fats include French fries and other fried fast food, some varieties of microwave popcorn, certain margarines and vegetable shortenings, packaged cakes and cookies, some pastries, and all processed foods that list partially hydrogenated vegetable oil on the label. SUMMARY Consuming artificial trans fats may increase inflammation and your risk of several diseases, including heart disease. 3. Vegetable and seed oils During the 20th century, the consumption of vegetable oils increased by 130% in the United States. Some scientists believe that certain vegetable oils, such as soybean oil, promote inflammation due to their very high omega-6 fatty acid content (30Trusted Source).

Although some dietary omega-6 fats are necessary, the typical Western diet provides far more than people need.

In fact, health professionals recommend eating more omega-3-rich foods, such as fatty fish, to improve your omega-6 to omega-3 ratio and reap the anti-inflammatory benefits of omega-3s.

In one study, rats fed a diet with an omega-6 to omega-3 ratio of 20:1 had much higher levels of inflammatory markers than those fed diets with ratios of 1:1 or 5:1 (31Trusted Source). However, evidence that a high intake of omega-6 fatty acids increases inflammation in humans is currently limited.

Controlled studies show that linoleic acid, the most common dietary omega-6 acid, does not affect inflammatory markers (32Trusted Source, 33Trusted Source).

More research is needed before any conclusions can be made.

Vegetable and seed oils are used as cooking oils and are a major ingredient in many processed foods. SUMMARY Some studies suggest that vegetable oil's high omega-6 fatty acid content may promote inflammation when consumed in high amounts. However, the

evidence is inconsistent, and more research is needed. 4. Refined carbohydrates

Carbohydrates have gotten a bad rap.

However, the truth is that not all carbs are problematic.

Ancient humans consumed high fiber, unprocessed carbs for millennia in the form of grasses, roots, and fruits (34Trusted Source).

However, eating refined carbs may drive inflammation (34Trusted Source, 35Trusted Source, 36Trusted Source, 37Trusted Source, 38Trusted Source).

Refined carbs have had most of their fiber removed. Fiber promotes fullness, improves blood sugar control, and feeds the beneficial bacteria in your gut.

Researchers suggest that the refined carbs in the modern diet may encourage the growth of inflammatory gut bacteria that can increase your risk of obesity and inflammatory bowel disease (34Trusted Source, 36Trusted Source).

Refined carbs have a higher glycemic index (GI) than unprocessed ones. High GI foods raise blood sugar more rapidly than low GI foods.

In one study, older adults who reported the highest intake of high GI foods were 2.9 times more likely to die of an inflammatory disease like chronic obstructive pulmonary disease (COPD) (37Trusted Source).

In a controlled study, young, healthy men who ate 50 grams of refined carbs in the form of white bread experienced higher blood sugar levels and increases in levels of a particular inflammatory marker (38Trusted Source).

Refined carbohydrates are found in candy, bread, pasta, pastries, some cereals, cookies, cakes, sugary soft drinks, and all processed foods that contain added sugar or flour.

SUMMARYHigh fiber, unprocessed carbs are healthy, but refined carbs raise blood sugar levels and promote inflammation that may lead to disease. 5. Excessive alcohol Moderate alcohol consumption has been shown to provide some health benefits.

However, higher amounts can lead to severe problems.

In one study, levels of the inflammatory marker CRP increased in people who consumed alcohol. The more alcohol they consumed, the more their CRP levels increased (39Trusted Source).

People who drink heavily may develop problems with bacterial toxins moving out of the colon and into the body. This condition — often called "leaky gut" — can drive widespread inflammation that leads to organ damage (40Trusted Source, 41Trusted Source).

To avoid alcohol-related health problems, intake should be limited to two standard drinks per day for men and one for women.

SUMMARYHeavy alcohol consumption may increase inflammation and lead to a "leaky gut" that drives inflammation throughout your body. 6. Processed meat Consuming processed meat is associated with an increased risk of heart disease, diabetes, and stomach and colon cancer (42Trusted Source, 43Trusted Source, 44Trusted Source).

Common types of processed meat include sausage, bacon, ham, smoked meat, and beef jerky.

Processed meat contains more advanced glycation end products (AGEs) than most other meats.

AGEs are formed by cooking meats and some other foods at high temperatures. They are known to cause inflammation (45Trusted Source, 46Trusted Source).

Of all the diseases linked to processed meat consumption, its association with colon cancer is the strongest.

Although many factors contribute to colon cancer, one mechanism is believed to be colon cells' inflammatory response to processed meat (47Trusted Source). SUMMARY Processed meat is high in inflammatory compounds like AGEs, and its strong association with colon cancer may partly be due to an inflammatory response. The bottom line Inflammation can occur in response to many triggers, some of which are hard to prevent, including pollution, injury, or sickness.

However, you have much more control over factors like your diet.

To stay as healthy as possible, keep inflammation down by minimizing your consumption of foods that trigger it and eating anti-inflammatory foods.

ADVERTISEMENT Customized meal planning that truly keeps you in mind

The 4 Your Health app customizes meal plans tailored to your specific needs. Share your favorite foods and weight loss goals. Cooking Light Diet then emails you meal plans, recipes, and grocery lists.

#### 14. What "food product" is not part of the USDA "My Plate" food program?

- a. Protein
- b. Fruit
- c. Vegetables
- d. Fats
- e. Dairy

What is MyPlate?

MyPlate is a reminder to find your healthy eating style and build it throughout your lifetime. Everything you eat and drink matters. The right mix can help you be healthier now and in the future. This means:

Focus on variety, amount, and nutrition. Choose foods and beverages with less saturated fat, sodium, and added sugars. Start with small changes to build healthier eating styles.

Support healthy eating for everyone.

Eating healthy is a journey shaped by many factors, including our stage of life, situations, preferences, access to food, culture, traditions, and the personal decisions we make over time. All your food and beverage choices count. MyPlate offers ideas and tips to help you create a healthier eating style that meets your individual needs and improves your health.

For a colorful visual of MyPlate and the 5 food groups, download What's MyPlate All About?.

U.S. DEPARTMENT OF AGRICULTURE

What is MyPlate? | ChooseMyPlate

<https://www.choosemyplate.gov/eathealthy/WhatIsMyPlate>

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Find your healthy eating style and maintain it for a lifetime Make half your plate fruits and vegetables: vary your veggies Make half your plate fruits and vegetables: focus on whole fruits

Make half your grains whole grains

Move to low-fat or fat-free milk or yogurt

Vary your protein routine

Make small changes

Take a look at A Brief History of USDA Food Guides to learn more about previous food guidance symbols. MyPlate Messages

Click on a message below to access tools that will help you share information from the Dietary Guidelines with your patients, clients, and peers.

#### Build a Healthy Eating Style

All food and beverage choices matter – focus on variety, amount, and nutrition.

Focus on making healthy food and beverage choices from all five food groups including fruits, vegetables, grains, protein foods, and dairy to get the nutrients you need.

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Eat the right amount of calories for you based on your age, sex, height, weight, and physical activity level. Building a healthier eating style can help you avoid overweight and obesity and reduce your risk of diseases such as heart disease, diabetes, and cancer.

Choose an eating style low in saturated fat, sodium, and added sugars.

Use Nutrition Facts labels and ingredient lists to find amounts of saturated fat, sodium, and added sugars in the foods and beverages you choose. Look for food and drink choices that are lower in saturated fat, sodium, and added sugar.

Eating fewer calories from foods high in saturated fat and added sugars can help you manage your calories and prevent overweight and obesity. Most of us eat too many foods that are high in saturated fat and added sugar. Eating foods with less sodium can reduce your risk of high blood pressure.

Make small changes to create a healthier eating style.

Think of each change as a personal “win” on your path to living healthier. Each MyWin is a change you make to build your healthy eating style. Find little victories that fit into your lifestyle and celebrate as a MyWin! Start with a few of these small changes.

Make half your plate fruits and vegetables.

Focus on whole fruits. Vary your veggies.

Make half your grains whole grains.

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Move to low-fat or fat-free milk or yogurt. Vary your protein routine.

Support healthy eating for everyone.

Create settings where healthy choices are available and affordable to you and others in your community. Professionals, policymakers, partners, industry, families, and individuals can help others in their journey to make healthy eating a part of their lives.

### 15. All healthy diets include:

- a. Grains
- b. Beans
- c. Organic choices
- d. Vegetable oils

**Organic Foods: What You Need to Know** Is organic food really healthier? Is it worth the expense? Find out what the labels mean and which foods give you the most bang for your buck.

What does “organic” mean? The term “organic” refers to the way agricultural products are grown and processed. While the regulations vary from country to country, in the U.S., organic crops must be grown without the use of synthetic pesticides, bioengineered genes (GMOs), petroleum-based fertilizers, and sewage sludge-based fertilizers. Organic livestock raised for meat, eggs, and dairy products must have access to the outdoors and be given organic feed. They may not be given antibiotics, growth hormones, or any animal by-products.

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**Organic vs. Non-Organic** Organic produce: Conventionally-grown produce: Grown with natural fertilizers (manure, compost). Grown with synthetic or chemical fertilizers. Weeds are controlled naturally (crop rotation, hand weeding, mulching, and tilling). Weeds are controlled with chemical herbicides. Pests are controlled using natural methods (birds, insects, traps) and naturally-derived pesticides. Pests are controlled with synthetic pesticides Organic meat, dairy, eggs: Conventionally-raised meat, dairy, eggs Livestock are given all organic, hormoneand GMO-free feed. Livestock are given growth hormones for faster growth, as well as non-organic, GMO feed. Disease is prevented with natural methods such as clean housing, rotational grazing, and healthy diet. Antibiotics and medications are used to prevent livestock disease. Livestock must have access to the outdoors. Livestock may or may not have access to the outdoors. The benefits of organic food How your food is grown or raised can have a major impact on your mental and emotional health as well as the environment. Organic foods often have more beneficial nutrients, such as antioxidants, than their conventionally-grown counterparts and people with allergies to foods, chemicals, or preservatives often find their symptoms lessen or go away when they eat only organic foods. Organic produce contains fewer pesticides. Chemicals such as fungicides, herbicides, and insecticides are widely used in conventional agriculture and residues remain on (and in) the food we eat. Organic food is often fresher because it doesn't contain preservatives that make it last longer. Organic produce is often (but not always, so watch where it is from) produced on smaller farms near where it is sold. Organic farming is better for the environment. Organic farming practices reduce pollution, conserve water, reduce soil erosion, increase soil fertility, and use less energy.

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Farming without pesticides is also better for nearby birds and animals as well as people who live close to farms. Organically raised animals are NOT given antibiotics, growth hormones, or fed animal byproducts. Feeding livestock animal byproducts increases the risk of mad cow disease (BSE) and the use of antibiotics can create antibiotic-resistant strains of bacteria. Organically-raised animals are given more space to move around and

access to the outdoors, which help to keep them healthy. Organic meat and milk are richer in certain nutrients. Results of a 2016 European study show that levels of certain nutrients, including omega-3 fatty acids, were up to 50 percent higher in organic meat and milk than in conventionally raised versions. Organic food is GMO-free. Genetically Modified Organisms (GMOs) or genetically engineered (GE) foods are plants whose DNA has been altered in ways that cannot occur in nature or in traditional crossbreeding, most commonly in order to be resistant to pesticides or produce an insecticide. Organic food vs. locally-grown food Unlike organic standards, there is no specific definition for "local food". It could be grown in your local community, your state, your region, or your country. During large portions of the year it is usually possible to find food grown close to home at places such as a farmer's market. The benefits of locally grown food Financial: Money stays within the local economy. More money goes directly to the farmer, instead of to things like marketing and distribution. Transportation: In the U.S., for example, the average distance a meal travels from the farm to the dinner plate is over 1,500 miles. Produce must be picked while still unripe and then gassed to "ripen" it after transport. Or the food is highly processed in factories using preservatives, irradiation, and other means to keep it stable for transport. Freshness: Local food is harvested when ripe and thus fresher and full of flavor. Small local farmers often use organic methods but sometimes cannot afford to become certified organic. Visit a farmer's market and talk with the farmers to find out what methods

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they use. Understanding GMOs The ongoing debate about the effects of GMOs on health and the environment is a controversial one. In most cases, GMOs are engineered to make food crops resistant to herbicides and/or to produce an insecticide. For example, much of the sweet corn consumed in the U.S. is genetically engineered to be resistant to the herbicide Roundup and to produce its own insecticide, Bt Toxin. GMOs are also commonly found in U.S. crops such as soybeans, alfalfa, squash, zucchini, papaya, and canola, and are present in many breakfast cereals and much of the processed food that we eat. If the ingredients on a package include corn syrup or soy lecithin, chances are it contains GMOs. GMOs and pesticides The use of toxic herbicides like Roundup (glyphosate) has increased 15 times since GMOs were introduced. While the World Health Organization announced that glyphosate is "probably carcinogenic to humans," there is still some controversy over the level of health risks posed by the use of pesticides. Are GMOs safe?

While the U.S. Food and Drug Administration (FDA) and the biotech companies that engineer GMOs insist they are safe, many food safety advocates point out that no long term studies have ever been conducted to confirm the safety of GMO use, while some animal studies have indicated that consuming GMOs may cause internal organ damage, slowed brain growth, and thickening of the digestive tract. GMOs have been linked to increased food allergens and gastro-intestinal problems in humans. While many people think that altering the DNA of a plant or animal can increase the risk of cancer, the research has so far proven inconclusive.

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Does organic mean pesticide-free? As mentioned above, one of the primary benefits of eating organic is lower levels of pesticides. However, despite popular belief, organic farms do use pesticides. The difference is that they only use naturally-derived pesticides, rather than the synthetic pesticides used on conventional commercial farms. Natural pesticides are believed to be less toxic, however, some have been found to have health risks. That said,

your exposure to harmful pesticides will be lower when eating organic. What are the possible risks of pesticides? Most of us have an accumulated build-up of pesticide exposure in our bodies due to numerous years of exposure. This chemical “body burden” as it is medically known could lead to health issues such as headaches, birth defects, and added strain on weakened immune systems. Some studies have indicated that the use of pesticides even at low doses can increase the risk of certain cancers, such as leukemia, lymphoma, brain tumors, breast cancer and prostate cancer. Children and fetuses are most vulnerable to pesticide exposure because their immune systems, bodies, and brains are still developing. Exposure at an early age may cause developmental delays, behavioral disorders, autism, immune system harm, and motor dysfunction. Pregnant women are more vulnerable due to the added stress pesticides put on their already taxed organs. Plus, pesticides can be passed from mother to child in the womb, as well as through breast milk. The widespread use of pesticides has also led to the emergence of “super weeds” and “super bugs,” which can only be killed with extremely toxic poisons like 2,4-Dichlorophenoxyacetic acid (a major ingredient in Agent Orange). Does washing and peeling produce get rid of pesticides?

Rinsing reduces but does not eliminate pesticides. Peeling sometimes helps, but valuable nutrients often go down the drain with the skin. The best approach: eat a varied diet, wash and scrub all produce thoroughly, and buy organic when possible.

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The best bang for your buck when shopping organic Organic food is often more expensive than conventionally-grown food. But if you set some priorities, it may be possible to purchase organic food and stay within your food budget. Know your produce pesticide levels Some types of conventionally-grown produce are much higher in pesticides than others, and should be avoided. Others are low enough that buying non-organic is relatively safe. The Environmental Working Group, a nonprofit organization that analyzes the results of government pesticide testing in the U.S., offers an annually-updated list that can help guide your choices. Fruits and vegetables where the organic label matters most

According to the Environmental Working Group, a nonprofit organization that analyzes the results of government pesticide testing in the U.S., the following fruits and vegetables have the highest pesticide levels so are best to buy organic: • Apples • Sweet Bell Peppers • Cucumbers • Celery • Potatoes • Grapes • Cherry Tomatoes • Kale/Collard Greens • Summer Squash • Nectarines (imported) • Peaches • Spinach • Strawberries • Hot Peppers Fruits and vegetables you DON'T need to buy organic

Known as the “Clean 15”, these conventionally-grown fruits and vegetables are generally low in pesticides.

- Asparagus • Avocado • Mushrooms • Cabbage • Sweet Corn • Eggplant • Kiwi • Mango
- Onion • Papaya • Pineapple • Sweet Peas (frozen) • Sweet Potatoes • Grapefruit • Cantaloupe

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Buy organic meat, eggs, and dairy if you can afford to While prominent organizations such as the American Heart Association maintain that eating saturated fat from any source increases the risk of heart disease, other nutrition experts maintain that eating organic grass-fed meat and organic dairy products doesn't carry the same risks. It's not the saturated fat that's the problem, they say, but the unnatural diet of an industrially-raised animal that includes corn, hormones, and medication. What's in American meat?

According to Animal Feed, conventionally raised animals in U.S. can be given: Dairy cows – antibiotics, pig and chicken byproducts, growth hormones, pesticides, sewage sludge Beef cows – antibiotics, pig and chicken byproducts, steroids, hormones, pesticides, sewage sludge Pigs – antibiotics, animal byproducts, pesticides, sewage sludge, arsenic-based drugs Broiler chickens – antibiotics, animal byproducts, pesticides, sewage sludge, arsenic-based drugs Egg laying hens – antibiotics, animal byproducts, pesticides, sewage sludge, arsenic-based drugs

Other ways to keep the cost of organic food within your budget Shop at farmers' markets. Many cities, as well as small towns, host a weekly farmers' market, where local farmers sell their produce at an open-air street market, often at a discount to grocery stores. Join a food co-op. A natural foods co-op, or cooperative grocery store typically offers lower prices to members, who pay an annual fee to belong

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Join a Community Supported Agriculture (CSA) farm, in which individuals and families join up to purchase "shares" of produce in bulk, directly from a local farm. Local and organic! Organic food buying tips Buy in season – Fruits and vegetables are cheapest and freshest when they are in season. Find out when produce is delivered to your market so you're buying the freshest food possible. Shop around – Compare the price of organic items at the grocery store, the farmers' market and other venues (even the freezer aisle). Remember that organic doesn't always equal healthy –Making junk food sound healthy is a common marketing ploy in the food industry but organic baked goods, desserts, and snacks are usually still very high in sugar, salt, fat, or calories. It pays to read food labels carefully. Why is organic food often more expensive?

Organic food is more labor intensive since the farmers do not use pesticides, chemical fertilizers, or drugs. Organic certification is expensive and organic feed for animals can cost twice as much. Organic farms tend to be smaller than conventional farms, which means fixed costs and overhead must be distributed across smaller produce volumes without government subsidies.

## 16. The benefits of fasting include:

- a. Weight loss
- b. Improved mitochondria function
- c. Regulates insulin
- d. Decreases cellular autophagy

Intermittent fasting: Surprising update Posted By Monique Tello, MD, MPH On June 29, 2018 @ 6:30 am In Diet and Weight Loss, Health | Comments Disabled

There's a ton of incredibly promising intermittent fasting (IF) research done on fat rats. They lose weight, their blood pressure, cholesterol, and blood sugars improve... but they're rats. Studies in humans, almost across the board, have shown that IF is safe and incredibly effective, but really no more effective than any other diet. In addition, many people find it difficult to fast.

But a growing body of research suggests that the timing of the fast is key, and can make IF a more realistic, sustainable, and effective approach for weight loss, as well as for diabetes prevention.

The backstory on intermittent fasting

IF as a weight loss approach has been around in various forms for ages, but was highly popularized in 2012 by BBC broadcast journalist Dr. Michael Mosley's TV documentary *Eat Fast, Live Longer* and book *The Fast Diet*, followed by journalist Kate Harrison's book *The 5:2 Diet* based on her own experience, and subsequently by Dr. Jason Fung's 2016 bestseller *The Obesity Code*. IF generated a steady positive buzz as anecdotes of its effectiveness proliferated.

As a lifestyle-leaning research doctor, I needed to understand the science. *The Obesity Code* seemed the most evidence-based summary resource, and I loved it. Fung successfully combines plenty of research, his clinical experience, and sensible nutrition advice, and also addresses the socioeconomic forces conspiring to make us fat. He is very clear that we should eat more fruits and veggies, fiber, healthy protein, and fats, and avoid sugar, refined grains, processed foods, and for God's sake, stop snacking. Check, check, check, I agree. The only part that was still questionable in my mind was the intermittent fasting part.

Intermittent fasting can help weight loss

IF makes intuitive sense. The food we eat is broken down by enzymes in our gut and eventually ends up as molecules in our bloodstream. Carbohydrates, particularly sugars and refined grains (think white flours and rice), are quickly broken down into sugar, which our cells use for energy. If our cells don't use it all, we store it in our fat cells as, well, fat. But sugar can only enter our cells with insulin, a hormone made in the pancreas. Insulin brings sugar into the fat cells and keeps it there.

Between meals, as long as we don't snack, our insulin levels will go down and our fat cells can then release their stored sugar, to be used as energy. We lose weight if we let our insulin levels go down. The entire idea of IF is to allow the insulin levels to go down far enough and for long enough that we burn off our fat.

Intermittent fasting can be hard... but maybe it doesn't have to be

Initial human studies that compared fasting every other day to eating less every day showed that both worked about equally for weight loss, though people struggled with the fasting days. So, I had written off IF as no better or worse than simply eating less, only far

more uncomfortable. My advice was to just stick with the sensible, plant-based, Mediterranean style diet.

Harvard Health Blog Intermittent fasting: Surprising update - H...

<https://www.health.harvard.edu/blog/intermittent-fasting-surpris...>

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New research is suggesting that not all IF approaches are the same, and some are actually very reasonable, effective, and sustainable, especially when combined with a nutritious plant-based diet. So I'm prepared to take my lumps on this one (and even revise my prior post).

We have evolved to be in sync with the day/night cycle, i.e., a circadian rhythm. Our metabolism has adapted to daytime food, nighttime sleep. Nighttime eating is well associated with a higher risk of obesity, as well as diabetes.

Based on this, researchers from the University of Alabama conducted a study with a small group of obese men with prediabetes. They compared a form of intermittent fasting called "early time-restricted feeding," where all meals were fit into an early eight-hour period of the day (7 am to 3 pm), or spread out over 12 hours (between 7 am and 7 pm). Both groups maintained their weight (did not gain or lose) but after five weeks, the eight-hours group had dramatically lower insulin levels and significantly improved insulin sensitivity, as well as significantly lower blood pressure. The best part? The eight-hours group also had significantly decreased appetite. They weren't starving.

Just changing the timing of meals, by eating earlier in the day and extending the overnight fast, significantly benefited metabolism even in people who didn't lose a single pound.

Why might changing timing help?

But why does simply changing the timing of our meals to allow for fasting make a difference in our body? An in-depth review of the science of IF recently published in *New England Journal of Medicine* sheds some light. Fasting is evolutionarily embedded within our physiology, triggering several essential cellular functions. Flipping the switch from a fed to fasting state does more than help us burn calories and lose weight. The researchers combed through dozens of animal and human studies to explain how simple fasting improves metabolism, lowering blood sugar; lessens inflammation, which improves a range of health issues from arthritic pain to asthma; and even helps clear out toxins and damaged cells, which lowers risk for cancer and enhances brain function. The article is deep, but worth a read!

So, is intermittent fasting as good as it sounds?

I was very curious about this, so I asked the opinion of metabolic expert Dr. Deborah Wexler, Director of the Massachusetts General Hospital Diabetes Center and associate professor at Harvard Medical School. Here is what she told me. "There is evidence to suggest that the circadian rhythm fasting approach, where meals are restricted to an eight to 10-hour period of the daytime, is effective," she confirmed, though generally she recommends that people "use an eating approach that works for them and is sustainable to them."

So, here's the deal. There is some good scientific evidence suggesting that circadian rhythm fasting, when combined with a healthy diet and lifestyle, can be a particularly effective approach to weight loss, especially for people at risk for diabetes. (However, people with advanced diabetes or who are on medications for diabetes, people with a history of eating disorders like anorexia and bulimia, and pregnant or breastfeeding women should not

attempt intermittent fasting unless under the close supervision of a physician who can monitor them.)

4 ways to use this information for better health

1. Avoid sugars and refined grains. Instead, eat fruits, vegetables, beans, lentils, whole grains, lean proteins, and healthy fats (a sensible, plant-based, Mediterranean style diet). 2. Let your body burn fat between meals. Don't snack. Be active throughout your day. Build muscle tone. 3. Consider a simple form of intermittent fasting. Limit the hours of the day when you eat, and for best effect, make it earlier in the day (between 7 am to 3 pm, or even

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<https://www.health.harvard.edu/blog/intermittent-fasting-surpris...>

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10 am to 6 pm, but definitely not in the evening before bed). 4. Avoid snacking or eating at nighttime, all the time.

17. The human gut (stomach, small intestine, large intestine, cecum) is BEST designed to digest:

- a. Animal products
- b. Vegetables
- c. Fruit
- c. Cellulose

Why is the paleolithic ketogenic diet (PKD) effective? Why is the PKD the effective diet? The paleolithic ketogenic diet is a healthy diet tailored to the biology of the human species regardless of age, gender, religion, skin colour, nationality, and country of origin. The paleolithic ketogenic diet is biologically the most fitting diet for humans, the deviation from which is the cause of almost all chronic medical conditions. Fortunately, the regenerative capacity of the human body is enormous, so if you have an established disease but you switch to our biological diet, the chances of recovery are good. In most cases, permanent recovery of patients occurs, and in some other cases, the condition may be stabilized followed by slow improvement.

How long does the recovery take? The extent and speed of recovery largely depend on the type of tissue affected by the disease.

The faster the cellular renewal and tissue replacement process is, the faster the healing process occurs. The fastest renewal rate is typical for skin and intestinal tissues (only 5-21 days), and accordingly, the regeneration of these tissues is the fastest. The regeneration of nerve tissues is the slowest, therefore, neurodegenerative diseases respond relatively slowly, which means that regeneration may take months or years.

For which age groups is the paleolithic ketogenic diet recommended? Contrary to common belief, the nutritional needs of humankind are not gender- or age-specific, therefore, the paleolithic ketogenic diet can be implemented at any age, from complementary feeding in infancy to old age.

Is the paleolithic ketogenic diet difficult? The paleolithic ketogenic diet does not cause any difficulties for the human body in biological terms, but due to the conditioned elements of our diet and our addictions to a previously unhealthy diet, psychological difficulties can be expected. This, however, has nothing to do with our biology. Our physiology and psychology must be treated separately. Our biological needs are given, and we cannot change that. Our psychological attitude, however, can be changed, and we need to change it, otherwise, we will not heal completely.

How fast is the diet? You don't have to wait for long for the first positive effects. The diet results in a spectacular improvement in weeks. When starting the diet, there is a transition period, which may be a few days, after which the positive effects will become obvious.

In case of which diseases is the paleolithic ketogenic diet effective? Below you'll find a list of diseases in case of which the paleolithic ketogenic diet proves to be effective. We have encountered with almost every medical condition. Yet, infectious and traumatic diseases, as well as surgical cases, fall into a separate category. Of course, there are always exceptions, rare diseases, and rare medical conditions. Therefore, in individual cases, we will only be able to respond in light of the full knowledge of the medical records. However, we can safely say that if you have a non-genetic chronic medical condition, we can either cure it or we can improve it significantly if you follow our guidelines.

Below you can read an extensive but not full list of the diseases we most often come across and treat with success:

- Metabolic syndrome conditions

Type 2 diabetes Obesity Hypertension

- Autoimmune diseases

Autoimmune thyroiditis (Hashimoto's thyroiditis) Crohn's disease Ulcerative colitis Eczema Psoriasis Rheumatoid arthritis Idiopathic thrombocytopenic purpura (ITP) Polycystic ovary syndrome (PCOS) Immune hepatitis Lupus Sarcoidosis Scleroderma Multiple sclerosis Wegener's granulomatosis and other granulomatous Myasthenia gravis Pernicious anemia Type 1 diabetes mellitus (T1DM) LADA diabetes Autoimmune encephalitis Anemia pernicious Antiphospholipid syndrome (APS) Aplastic anemia Temporal arteritis Celiac disease, Guillain-Barré syndrome (GBS) Hashimoto's thyroiditis Graves' disease Myasthenia gravis Pemphigus vulgaris Primary biliary cirrhosis (PBC) Sjogren's syndrome Multiple sclerosis (MS) Ankylosing spondylitis (Bechterew's disease) Systemic Lupus Erythematosus (SLE) Wegener's granulomatosis Lichen plans Alopecia universalis Behcet's disease Neuromyotonia

- Cancers

Rectal cancer Colon cancer Hodgkin's lymphoma Non-Hodgkin's Lymphoma Chronic lymphocytic leukemia Acute lymphoblastic leukemia Renal cancer Liver cancer Brain cancer Melanoma Breast cancer Cervical intraepithelial neoplasia (CIN) Thyroid cancer Lung cancer Bronchial cancer Larynx cancer Soft palate cancer Pancreatic tumor Biliary tumors Esophagus cancer Prostate cancer Metastatic cancer

- Other Conditions

Sleep apnea Sleep disorders Restless legs syndrome (RLS) PCOS syndrome Female infertility Male infertility Transient global amnesia Pickwick syndrome Toxicosis of pregnancy Gestational diabetes Coronary artery bypass surgery Paranoid schizophrenia Panic disorder Gingivitis Hyperactivity, ADHD Autism Gilbert's syndrome Chronic fatigue syndrome (CFS) Migraine Allergy Reflux Breast and ovary cysts Epilepsy (generalized epilepsy, localization-related epilepsy, other forms of epilepsy) Depression Anxiety Learning difficulties Dry eye syndrome Myopathy Prostate hypertrophy Renal failure All forms of anemia Upper respiratory tract infections Leg ulcers Uncertain abdominal complaints Cystic diseases Overmedicated conditions

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Isotopic biogeochemistry ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) of fossil vertebrate collagen: application to the study of a past food web including Neandertal man

The C/N ratio and amino acid composition of organic matter extracted from fossil mammal bones from the Paleolithic site at Marillac (Charentes, France) shows that this organic matter comes from collagen.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of known-diet fossil species demonstrate that these values have been preserved through fossilization processes, and that these fossil mammals can be used as ecological references to determine the Neandertal position in the past food web. Initial Neandertal  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values suggest that he was mostly carnivorous.

Les rapports C/N et le spectre d'acides aminés de la matière organique extraite des ossements de mammifères fossiles du site de Marillac (Charentes, France) montrent que cette matière organique provient du collagène. Les valeurs de  $\delta^{13}\text{C}$  et de  $\delta^{15}\text{N}$  de mammifères fossiles dont le régime alimentaire est connu montrent que ces valeurs n'ont pas été affectées par la fossilisation et donc ces mammifères fossiles peuvent servir de standards écologiques pour replacer l'homme de Neandertal dans son réseau trophique. Les premières essentiellement carnivores. Les valeurs de  $\delta^{13}\text{C}$  et de  $\delta^{15}\text{N}$  mesurées pour cet homme suggèrent qu'il était

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Introduction

Determination of the diet of fossil vertebrates is often a difficult problem. Most of the available information is provided by a study of the digestive apparatus remains, mainly teeth and jaws; so the feeding behaviour of an animal can be defined by comparisons with the anatomy of similar recent species and by a study of the jaw mechanisms. But only certain types of food can be deduced from this type of study, and for most fossil species, it is not easy to answer the apparently simple question: "What did it eat?" In exceptional cases when the stomach contents are preserved (Krausel, 1922; Pollard, 1968; Koenigswald, 1980) identification of the food actually eaten by the animal is possible, but his last meal does not necessarily reflect the normal diet of the animal. Dental wear marks on tooth enamel can provide information about food hardness (Grine & Kay, 1988; Van Valkenburgh, 1988; Ussunet, 1990) and about the possibilities of feeding resources. However, these methods do not give accurate qualitative information about the diet and the dietary variations of an individual within a population, or of a population within a species. For that reason, isotopic biogeochemistry can provide an important contribution, and through animal diet we can make a picture of the environment.

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$^{13}\text{C}$  and  $^{15}\text{N}$  as palaeodietary tools

$^{13}\text{C}$  and  $^{15}\text{N}$  isotopic compositions of animal organic tissues are linked to those of diet (DeNiro & Epstein, 1977, 1981): stable-carbon isotope abundance ratios are similar to those of diet, but there is a significant isotopic enrichment for  $^{15}\text{N}$  between diet and animal tissues. As different categories of feeding resources can be distinguished by  $^{13}\text{C}$  and  $^{15}\text{N}$  natural isotope abundances, vertebrates can be replaced in food webs using some of their

organic compounds, such as bone collagen which can survive after an animal's death (Schoeninger & DeNiro, 1984; Schoeninger, 1985). In collagen, carbon isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ ) reflect the corresponding isotope ratios in the plants at the base of food web, mainly determined by the environment (marine or aerial) where photosynthesis occurs and, for terrestrial environment, by the type of photosynthetic pathway: C<sub>3</sub>, C<sub>4</sub>, or CAM (Bender, 1968; Smith & Epstein, 1971; Deines, 1980; O'Leary, 1981). DeNiro & Epstein (1977) show that, if there is very little difference in isotopic composition between diet and animal tissues as a whole, collagen is specifically enriched by almost 5‰ in  $^{13}\text{C}$  compared to diet. On the other hand, by means of successive isotopic fractionations, isotope abundance ratios systematically enrich in  $^{15}\text{N}$  when following the food web (Minagawa & Wada, 1984). It is thus possible to distinguish marine from terrestrial animals, herbivorous from carnivorous animals, browsers (C<sub>3</sub>-plants eaters) from grazers (C<sub>4</sub>-plants eaters) in Savannah landscapes. Nowadays, this method is currently used in ecology (DeNiro & Epstein, 1978; Ambrose & DeNiro, 1986a; Tieszen & Boutton, 1988; Van der Merwe et al., 1990; Vogel et al., 1990) and archaeology by the study of collagen preserved in ancient bones (Van der Merwe & Vogel, 1978; Tauber, 1981; Van der Merwe et al., 1981; Chisholm et al., 1983; Boutton & Klein, 1984; Hopson & Collier, 1984; Ambrose & DeNiro, 1986b; Sealy & Van der Merwe, 1986; Walker & DeNiro, 1986; DeNiro, 1987; Noe-Nygaard, 1988). As organic matter derived from bone collagen has been extracted from very old fossil bones (Heller, 1965; Wyckoff, 1969, 1972, 1974; Davidson et al., 1978) it was tempting to test this methodological approach to similar organic matter. Collagen was extracted from fossil vertebrates of different stratigraphic ages to determine if it preserved its original isotopic composition. In this work, we present the results obtained from Pleistocene mammals from Marillac. The preliminary results concerning Neandertal has to be seen within the context of a single time and place.

#### Materials

Bones of recent vertebrates were collected in the field or sampled from the comparative anatomy collections of the Laboratoire de Paléontologie des Vertébrés et de Paléontologie humaine of the University Pierre et Marie Curie (Paris). The fossil samples studied came from the excavations at the prehistoric site of Marillac (Charentes, France) directed by B. Vandermeersch in collaboration with B. Lange-Badre. This site is located a few kilometers southeast from the town of La Rochefoucauld, on the right side of the Ligogne, a tributary of the Tardoire. Discovered in the 1930s during the excavation of a pit in Jurassic limestone, it is a collapsed-roof cave, almost 15 m in diameter, and prolonged laterally by a small shelter and a gallery network. This system, of karstic origin, is connected with the stream of the Ligogne which frequently flooded the site, altering the sedimentation of the silty parts and the conservation of the fossil remains. Of the 12 stratigraphic levels distinguished from the surface: the first 11 have yielded a Mousterian

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Table 1 Isotopic compositions (‰ vs PDB, 615‰ vs atmospheric dinitrogen) of recent cow bone and tooth collagen, with or without acetone treatment

cow (*Bos taurus*),  $\delta^{13}\text{C}$  ‰

Bone - 22.0 Bone - 22.1 Bone - 22.0 Bone - 22.1 Bone - 21.9  $\delta^{15}\text{N}$  ‰ - 22.0 s.d. 0.1 Acetone-treated bone - 22.0 Tooth - 21.9 Tooth - 22.0  $\delta^{13}\text{C}$  ‰ - 22.0 s.d. 0.1 Acetone-treated tooth - 21.9  
+5.1 +5.6 +5.2 +5.2 +5.2  $\delta^{15}\text{N}$  ‰ f5.3 0.2 f5.1 f.i.1 +5.1 f5.1  
+5.1

lithic industry of the Quina type (Vandermeersch, 1965; Meignen & Vandermeersch, 1986) and a homogeneous fauna relatively common for this period. Among the macrofauna, reindeer (*Rangifer tarandus*), auroch (*Bos primigenius*), horse (*Equus caballus germanicus*), marmot (*Marmota marmota*), fox (*Vulpes vulpes*), hyena (*Crocuta crocuta spelaea*), and wolf (*Canis lupus*) have been identified. Layers 9 and 10 have also yielded human remains from a Neandertal (*Homo sapiens neandertalensis*). Fauna, lithic industry and the presence of *Homo neandertalensis* date Marillac site from the end of the Early Weichselian (about -40,000 years).

#### Collagen extraction and analysis

Collagen extraction has been completed on bone or tooth fragments of each species. For the Neandertal sample, a little cranial fragment damaged and unconnected with other skull remains, was used. Some of these samples had not been glued whereas others had been glued with Rhodopas (polyvinyl acetate: synthetic glue, soluble in acetone). Samples were cleaned by scratching, sandblasting, sonication in distilled water (or acetone for glued samples), and powdered to less than 0.7 mm. In the case of glued samples, visible glue traces were eliminated, samples plunged into acetone and rinsed several times, and crushed into smaller pieces after each rinsing. Finally, the powder was carefully rinsed several times with distilled water until no white precipitate occurred when mixed with water, and dried; this procedure removed the glue. To check whether acetone changes the isotopic values of collagen, isotopic analyses were made on recent cow samples of known isotopic composition treated in the same way as the glued fossils. The results (Table 1) show that acetone treatment does not change isotopic compositions in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . Moreover, isotopic compositions of collagen extracted from the non-glued auroch bone (3101) and the glued and acetone treated auroch bone (4001, 4002, 4003) are almost identical (Table 2). We can thus conclude that treatment with synthetic glues does not preclude isotopic analyses on bones.

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Treatment followed that described by DeNiro & Epstein (1981) with some modifications (Bocherens et al., 1988): the sample (generally 0.5 g for recent specimens and 0.6 to 2.5 g for fossil ones) is decalcified in 1 M HCl for 20 min at room temperature, and filtered through a MF-Millipore 5  $\mu\text{m}$  filter. The residue, containing collagen and, in the case of some fossils, minerals insoluble in HCl, is plunged into 0.125 N NaOH for 20 hr at room temperature. After rinsing with distilled water by filtration or repeated centrifugations, this residue is plunged into 10<sup>-2</sup> M HCl (pH = 2) in closed tubes, at 100°C for 17 hr, to solubilize the collagen. More collagen is solubilized at pH 2 than at pH 3 (as used by DeNiro & Epstein, 1981), without any change in carbon and nitrogen isotopic compositions. After centrifugation of the tubes at 6300 r.p.m., the supernatant (containing solubilized collagen) is lyophilized and analysed for its isotopic composition following a modified Dumas method. Carbon and nitrogen isotope compositions were measured on  $\text{CO}_2$  and  $\text{N}_2$ , obtained by combustion of about 5 mg of collagen in a quartz sealed tube with  $\text{CuO}$  at different dwell times of temperature, the first (and the higher) being 850°C. After breaking the tube in a vacuum, the evolved  $\text{CO}_2$  and  $\text{N}_2$  are purified and analysed on a mass spectrometer (Finnigan Delta E or VG Sira 9) fitted with multiple ion collectors and dual inlet system equipped for rapid switching between reference and sample. Isotopic abundances measured in this way are relative abundances: enrichment or depletion of heavy isotopic varieties ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) are measured vs. international standards. The isotope ratios are

expressed for carbon as  $\delta^{13}\text{C}$  vs. PDB (a marine carbonate) and for nitrogen as  $\delta^{15}\text{N}$  vs. atmospheric N, (Mariotti, 1984):

$$\delta^{13}\text{C}\text{‰} = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \quad \delta^{15}\text{N}\text{‰} = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where R stands for isotope ratios:  $^{13}\text{C}/^{12}\text{C}$ ;  $^{15}\text{N}/^{14}\text{N}$ . Total organic C and N of each organic matter sample were determined by volumetric analysis of evolved gases ( $\text{CO}_2$  and  $\text{N}_2$ ). Analytical precisions, determined as the standard deviation of 6 values obtained for > 15 different combustions of the same sample ("Sigma insoluble collagen, type I from bovine Achilles tendon"), were 0.1‰ for  $\delta^{13}\text{C}$  and 0.1‰ for  $\delta^{15}\text{N}$ . This reproducibility included the heterogeneity of this commercial collagen which is inserted as internal standard in each of our sample isotope analysis series. Extractions performed independently on the same bone powder (recent or fossil) show the same reproducibilities of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values: our extraction method does not introduce any isotopic fractionation in the whole process. The amino acid spectrum of organic matter samples extracted from our fossils have been compared to the spectrum of collagen extracted from recent bone following the same process (Figure 1). Indeed, it is well known that collagen has a particular amino acid composition (and sequence).

#### Results

These are shown in Table 2 for fossil mammals and in Table 3 for recent mammals. All the analysed fossil samples have provided organic matter with this extraction method. None the

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n Fox (recent)

0 Horse (recent)

300

250

7 ap

“, 200 D 8 [L 150

ffl Auroch (Marillac)

U Horse (Marillac) q Neandertal (Marillac)

0

Hyp Asp Thr Ser Glu Pro Gly Ala Val h4et lieu Leu Tyr Phe Hyl Lys His Arg

Figure 1. Comparison of amino acid spectrum from recent fox and horse collagen and fossil auroch (sample no. 4002), horse (sample no. 3601) and Neandertal (sample no. 27801) organic matter from Marillac.

less, extraction yields are variable, between 6.7 and 78 mg . g<sup>-1</sup> of the fossil sample, which represents approximately 3-40% of the collagen present in a fresh bone (approximately 204-39 mg . g<sup>-1</sup>, mean value calculated from 52 extractions performed in our laboratory on recent bones). These variations of yield between different samples are not due to the extraction method, as three extractions independently performed on the same fossil bone powder of an auroch bone gave very similar yields (respectively: 33.2, 34.4 and 35.0 mg g<sup>-1</sup>; see extractions nos 4001, 4002 and 4003 on Table 2). This heterogeneity of the "collagen" content in fossil bones had already been noticed by other authors working on fossil bone biochemical composition (e.g., Davidson et al., 1978). We use "collagen" to underline the fact that the completeness of the molecule cannot be ascertained by the present work. Pressure values of the gas measured after cryogenic separation of  $\text{CO}_2$  and  $\text{N}_2$ , are in the

same scale of variation as those measured for recent collagen samples, so the organic matter extracted from our fossils presents C/N ratios similar to those of collagen extracted from recent vertebrates. DeNiro (1985) emphasized the importance of this parameter: collagen C/N ratio is near 3 and only samples with this C/N ratio have preserved their isotopic compositions. Moreover, amino acid analysis of the organic matter extracted from some animal bones from the site at Marillac show a perfect identity with the collagen extracted from a recent animal (Figure 1): the proportion of glycine residues is about one-third, proline is present at about 12%, an important quality in comparison with other proteins, and the two amino acids found almost exclusively in collagen, hydroxyproline and hydroxylysine, are present. This emphasizes the fact that extracted organic matter comes only from collagen and suggests, in association with the values of C/N ratios, that it must be the same for the other samples from the site at Marillac. The Neandertal amino acid spectrum presents a depletion in glycine, but shows hydroxyproline and hydroxylysine percentages similar to those of collagen from recent bones. Enrichment in aspartic acid and valine in comparison to

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Table 2 Extraction yields and isotopic compositions (PC, PN) of Marillac fossils (4001,4002 pnd 4003) represent three extractions independently performed OP the same bone Species

Collagen No	of content	6°C	6"N	extractions	!mg.g ' )	%O	%O
Reindeer (Ran&r tarandus)	Reindeer (Rangifer tarandus)	Reindeer (Ran&k tarandus)	Reindeer (Rangi& tarandus)	Reindeer (Rangifer tarandus)	Auroch (Bosprimigtnius)	Auroch (Bosprimigeniu)	Auroch (Bos primigemus)
Adroch (Bosprim~g~~~ius)	Horse (Equus cab&u germanicus)	Horse (Equus caballus germanicus)	Horse (Equus caballusgermanicus)	Marmot (Marmota marmota)	Hyena (Crocukz crocuta sppaelea)	Wolf (C&s lupus)	Fox ( c'u1pe.r uulpes)
Neanderthal Man (Homo sapiens neandertalensis)	3221 9 - 20.2 +0.9	3901 24 - 19.7 f3.1	392 1 17 - 19.9 f3.3	27 27 -20.1 +tS1	3201 56 - 19.0 +3.5	3101 20 -20.6 +63	4001 33.2 -20.1 f6.3
4002' 35 - 20.0 f6.4	4003 34.4 - 20.0 i-6.3	3601' 33 -20.5 f5.0	42,00 1 9 - 20.8 +4,8	42,30 I 6.7 - 20.5 +6,0	3701 25 -21.8 +4,0	3301 12 - 19.5 + 10.3	3501 78 - 18.5 +9.6
3801 77 - 20.0 +8.6	27,801' 7 - 20.2 f9.3	'Samples chemically analysed for amino-acids. All samples are from bones, except 3101, which is from a tooth.					

Table 3 Isotopic compositions (S'S, S"N) of recent European mammals collagen

Species

Origin of the sample

cow (Bos taurus) Pyrknkes (France)	cow (Bos taurus) Nikvre (France)	cow (Bos taurus) FraIEc	Horse (Equus cab&s) Quercy (France)	Sheep (Ouis ark) PyrknPes (France)	Chamois (Rupicapra rupicapra) Pyrtnies (France)	Chamois (Rupicapra rupicapra) Pyrtnkes (France)	Chamois (Rupkpra rupicapra) Pyrtnies (France)	Marmot (Marmota marmota) Alps (France)	Common genet (Genetta genetta) Aveyron (France)	Lynx (&u &) Lithuania	Wolf (Canis 1tqus) Russia	Wolf (Canis lupus) France	Common otter (Lutra lutra) Toulouse (France)
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-21.9	-21.1	-21.3	-21.4	-22.1	- 20.3	- 20.3	- 19.9	-21.5	- 20.0	-21.7	- 18.5	- 17.0	- 19.8
f5.2	f5.6	+4.7	+2.6	+4.8	+2.5	+2.8	+3.0	+ 1.4	+ 10.8	+ 7.9	+ 10.2	+ 10.0	+13.4

collagen may be due to the contribution of non-collagenous bone proteins (amino acid values for these proteins are presented by Hare, 1980), since the yield in collagen was low for Neandertal bone.

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Table 4 Amino acid compositions of recent collagen and organic matter extracted from fossil bones of Marillac (expressed per thousand residues)

Amino acid

FOX (recent)

HOW? (recent)

AUIYXh (Marillac)

HOW (Marillac)

Neandertal (Marillac)

HYP 92 75 96 88 9 Asp 45 49 43 48 81 Thr 21 19 16 18 31 SC1 36 40 30 32 56 Glu 75 82 74 77 88 Pro 124 144 124 123 175 Gly 337 304 352 354 211 Ala 107 116 118 112 82 Val 22 25 21 20 39 &let 3 2 2 2 1 Ilru 12 11 II 11 15 LCU 27 29 24 24 31 'T'yr 3 1 0 0 0 Phc 14 I5 12 I1 15 Hyl 7 3 5 6 4 LYS 23 29 26 26 28 His 4 2 1 2 I A% 48 52 46 47 44

All the  $\delta^{13}C$  values obtained in this site are between -21.8 and -18.5‰ (mean+s.d.: -20.3 + 0.7‰ for herbivorous species and -19.3 + 0.8‰ for carnivorous species). This enrichment between carnivorous species and herbivorous species is not statistically significant, but it can be noted that the same observation has been made by Price et al. (1985) on

recent samples.  $\delta^{15}N$  values of herbivorous species are between 0.9 and 6.3‰ (mean + s.d.: +4.5 + 1.7‰), whereas those of carnivorous species are significantly higher, between 8.6 and 10.5‰ (mean+s.d.: +9.5 + 0.9‰). There is a mean enrichment of +5.2‰ between herbivorous and carnivorous species, very similar to the enrichment of 5.7‰ measured by Ambrose & DeNiro (1986a) from mammal fauna of the basin of Naivasha in East Africa.

#### Discussion

On a diagram of  $\delta^{13}C$  vs.  $\delta^{15}N$  (Figure 2)) the points representative of fossil herbivorous and carnivorous animals are well separated. Herbivorous animals from the Marillac site show a relatively important  $\delta^{15}N$  variability, and this variability does not appear to be linked to the collagen yield for a given species (on Table 2 we observe close values of  $\delta^{15}N$  for very different extraction yields of "collagen" from horse and reindeer samples). This does not confirm the suggestion that samples with low yields in "collagen" are  $\delta^{15}N$ -enriched relative to their original isotopic values (Tuross et al., 1988). Too few samples are available to find any trends in these variations according to the different species. However, the analysis of the two aurochs samples show higher  $\delta^{15}N$  values than those from other herbivores. This variability may be linked to climatic changes between different stratigraphic levels, from which individuals are derived. Sedimentological analysis showed that cold and dry phases alternated with wet and warmer periods (Debenath, 1975) and it is known that variations of aridity generate

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Figure 2.  $\delta^{15}N$  vs.  $\delta^{13}C$  diagram of Marillac mammals collagen variations in  $\delta^{15}N$  values (Ambrose & DeNiro, 1986; Heaton et al., 1986; Sealy et al., 1987). It is also possible that different environments coexisted in the proximity of Marillac and animals brought back by Neandertals to their territory may have been caught at some

distance from the site. Another possibility is that herbivores with higher  $\delta^{15}\text{N}$  values are young animals, not yet weaned as breast-feeding is known to enrich collagen in  $^{15}\text{N}$ : a diet of mother's milk acts in the same way as a carnivorous diet for  $^{15}\text{N}$  enrichment (Fogel et al., 1989). A more exhaustive study of these variations of  $\delta^{15}\text{N}$  values among Marillac herbivores may reveal palaeoenvironmental variations. Among the three species of carnivores analysed from Marillac, fox, the least carnivorous of these (Schilling et al., 1983), is the least enriched in  $^{15}\text{N}$ , whereas hyena, which exclusively consumes meat (Vereshchagin & Baryshnikov, 1984), shows the highest  $\delta^{15}\text{N}$  value. The wolf  $\delta^{13}\text{C}$  value is less negative than for other carnivores from the site, and is discussed later. A comparison of isotopic values from the site at Marillac with values of recent European mammals (Table 3) show that the isotopic values of recent and Marillac herbivores overlap well (Figure 3); the same observation can be made for the carnivores.  $\delta^{13}\text{C}$  values of recent herbivores seem more negative than for those at Marillac, but this difference is not easy to explain since the majority of recent samples are not from wild animals and their diet may have been influenced by anthropic factors.  $\delta^{13}\text{C}$  values of recent and subrecent reindeer collagen published by Nelson et al. (1986) show variations on a scale comparable to those from Marillac reindeers we analysed: - 19.0 to - 20.6‰ (against - 19.8 to - 21.3‰ in Nelson et al., 1986).  $\delta^{15}\text{N}$  values published in the same work fit only with our lowest values (between + 1.0 and + 3.8‰). As observed from the prehistoric site of Marillac, recent European wolves seem to be enriched in  $^{13}\text{C}$  compared to other carnivores. This difference between wolves and other carnivores is not clearly understood, since wolves do not seem to select specific parts of their prey; once wolves have fed on a large animal, usually only bones and hair are left (Mech, 1970). Such a difference cannot be attributed either to food of freshwater origin, since the  $^{13}\text{C}$  isotope composition of freshwater otter (which eats freshwater fishes and small vertebrates) is comparable to the  $\delta^{13}\text{C}$  value of small terrestrial carnivores and suggests that a freshwater diet does not change  $\delta^{13}\text{C}$  values in carnivores.

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Figure 3.  $\delta^{13}\text{C}$  vs. mammals.

12

1

10

8

6

4

2

q Herbivores

2-

$\delta^{15}\text{N}$  diagram of collagen from Marillac mammals compared to recent European

But the  $\delta^{15}\text{N}$  value for otter is clearly higher than that for terrestrial carnivores, which agrees with longer food chains in aquatic ecosystems and consequently, more successive  $^{15}\text{N}$ -enrichments. Other recent European carnivores show  $\delta^{15}\text{N}$  values comparable to those of Marillac carnivores. Marillac mammals belonging to species whose diet is known by recent representatives, yield isotopic values similar to these recent representatives. Thus they are "ecological standards" usable as reference values for the study of Marillac Neandertal's place within the food web. The  $\delta^{15}\text{N}$  value of this man places him among the

carnivores. But since the amino acid composition of organic matter extracted from Neandertal bone is depleted in glycine compared to modern collagen, this may have an influence on isotopic values (Tuross et al., 1988). These authors have shown that glycine depletion tends to give higher values, since the  $\delta^{15}\text{N}$  value is about 5‰ lower than whole collagen. In the Neandertal sample, the glycine value is 21‰ instead of about 30‰ in collagen. It should be possible to estimate the over-estimation of  $\delta^{15}\text{N}$  value due to glycine depletion. A simple calculation, considering the loss of 100‰ of glycine in comparison to fresh collagen and the participation of this amino acid for 30% of total nitrogen in collagen, gives a  $\delta^{15}\text{N}$  value about 0.7‰ lower than the measured value. The corrected value of +8.6‰ is still within the range of variation found in carnivorous mammals. This Neandertal appears to be a meat-consumer, hunting mammals for food, a conclusion confirmed by cut marks found several times on the bones of some species at the site. The question arises about the possibility of this man travelling to the ocean coast (almost 120 km today, probably a bit more 40,000 years ago) and possibly eating a marine diet, at least for part of the year; but his  $\delta^{13}\text{C}$  value does not sustain this hypothesis: the  $\delta^{13}\text{C}$  value should be less negative than for the terrestrial species from the site and this is not the case. Moreover, the presence of man during the whole year in the layer 9 (which provided the analysed human remains) is attested by the slaughter pattern of the reindeer. These preliminary results on the Neandertal sample have to be corroborated by further analyses. Until now, application of this methodology was limited to sites younger than 10,000 years old, since no older sites with collagen-rich fossils had been studied. Several protein extractions

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had been attempted from fossils older than 10,000 years, but even when a collagenic residue had been isolated, yields were by far lower than ours (Cohen-Solal et al., 1987; Davidson et al., 1978; Wyckoff, 1972). Only 30,000-year-old cave bears (Bocherens et al., 1990) and a 70 million-year-old dinosaur supplied organic matter with a collagen composition and a yield high enough to allow isotopic studies with palaeodietary implications (Bocherens et al., 1988). Previous results had led to the general assumption that bone collagen undergoes very large losses in the first steps of burial and diagenesis (Cohen-Solal et al., 1987) even if few remains could persist for a very long time. Yields obtained with Marillac fossil mammals demonstrate that there are exceptions, since it was possible to find in some samples more than one third of the initial collagen. It would be very useful to determine what fossilization conditions allow good collagen conservation.

Conclusions

The results obtained with the Marillac fossil mammals demonstrate that this methodology can be used for fossils older than 10,000 years: organic matter coming exclusively from collagen may be preserved with its original isotopic composition in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

Moreover, isotopic values reliability does not seem to be linked to the yield in collagen in these fossils. Since isotopic composition in  $\delta^{15}\text{N}$  may be influenced by factors other than diet, it is necessary to obtain isotopic values from several representatives of the food web, some of known diet, in order to infer any palaeodietary conclusions. None the less, in the case of known-diet species, isotopic values may provide valuable information about palaeoenvironmental parameters, such as aridity.

New fossils from Jebel Irhoud, Morocco and the pan-African origin of *Homo sapiens* Jean-Jacques Hublin<sup>1,2</sup>, Abdelouahed Ben-ncer<sup>3</sup>, Shara e. Bailey<sup>4</sup>, Sarah e. Freidline<sup>1</sup>, Simon neubauer<sup>1</sup>, Matthew M. Skinner<sup>5</sup>, Inga Bergmann<sup>1</sup>, Adeline Le Cabec<sup>1</sup>, Stefano Benazzi<sup>6</sup>, Katerina Harvati<sup>7</sup> & Philipp Gunz<sup>1</sup>

Fossil evidence points to an African origin of *Homo sapiens* from a group called either *H. heidelbergensis* or *H. rhodesiensis*. However, the exact place and time of emergence of *H. sapiens* remain obscure because the fossil record is scarce and the chronological age of many key specimens remains uncertain. In particular, it is unclear whether the present day 'modern' morphology rapidly emerged approximately 200 thousand years ago (ka) among earlier representatives of *H. sapiens*<sup>1</sup> or evolved gradually over the last 400 thousand years<sup>2</sup>. Here we report newly discovered human fossils from Jebel Irhoud, Morocco, and interpret the affinities of the hominins from this site with other archaic and recent human groups. We identified a mosaic of features including facial, mandibular and dental morphology that aligns the Jebel Irhoud material with early or recent anatomically modern humans and more primitive neurocranial and endocranial morphology. In combination with an age of  $315 \pm 34$  thousand years (as determined by thermoluminescence dating)<sup>3</sup>, this evidence makes Jebel Irhoud the oldest and richest African Middle Stone Age hominin site that documents early stages of the *H. sapiens* clade in which key features of modern morphology were established. Furthermore, it shows that the evolutionary processes behind the emergence of *H. sapiens* involved the whole African continent. In 1960, mining operations in the Jebel Irhoud massif 55 km southeast of Safi, Morocco exposed a Palaeolithic site in the Pleistocene filling of a karstic network. An almost complete skull (Irhoud 1) was accidentally unearthed in 1961, prompting excavations that yielded an adult braincase (Irhoud 2)<sup>4</sup>, an immature mandible (Irhoud 3)<sup>5</sup>, an immature humeral shaft<sup>6</sup>, an immature ilium<sup>7</sup> and a fragment of a mandible<sup>8</sup>, associated with abundant faunal remains and Levallois stone-tool technology<sup>6</sup>. Although these human remains were all reported to come from the bottom of the archaeological deposits, only the precise location of the humeral shaft was recorded. The interpretation of the Irhoud hominins has long been complicated by persistent uncertainties surrounding their geological age. They were initially considered to be around 40 thousand years (kyr) old and an African form of Neanderthals<sup>9</sup>. However, these affinities have been challenged<sup>5,10,11</sup> and the faunal<sup>8</sup> and microfaunal<sup>12</sup> evidence supported a Middle Pleistocene age for the site. An attempt to date one of the hominins directly by uranium series combined with electron spin resonance (U-series/ESR)<sup>3</sup> suggested an age of  $160 \pm 16$  kyr (ref. 13). Consistent with some genetic evidence<sup>14</sup>, fossils from Ethiopia (Omo Kibish is considered to be as old as approximately 195 kyr (ref. 15) and Herto has been dated to approximately 160 thousand years ago (ka)<sup>16</sup>) are commonly regarded as the first early anatomically modern humans (EMH). Notably, Omo Kibish 1 and the Herto specimens appear to be more derived than the supposedly contemporaneous or even younger Irhoud hominins. It has therefore been suggested that the archaic features of the Irhoud fossils may indicate that north African *H. sapiens*

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a  
b

Figure 1 | Facial reconstruction of Irhoud 10. a, b, Frontal (a) and basal (b) views. This superimposition of Irhoud 10 (beige) and Irhoud 1 (light blue) represents one possible alignment of the facial bones of Irhoud 10. Nine alternative reconstructions were included in the statistical shape analysis of the face (see Methods and Fig. 3). The maxilla, zygomatic bone and supra-orbital area of Irhoud 10 are more robust than for Irhoud 1. Scale bar, 20 mm.

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interbred with Neanderthals<sup>17</sup>, or that the Irhoud hominins represented a north African, late surviving, archaic population<sup>18</sup>. New excavations at Irhoud have enabled the recovery of in situ archaeological material and the establishment of a precise chronology of the deposits, which are much older than previously thought<sup>3</sup>. The excavation yielded a new series of hominin remains, including an adult skull (Irhoud 10) comprising a distorted braincase and fragments of the face (Fig. 1), a nearly complete adult mandible (Irhoud 11) (Fig. 2), one maxilla, several postcranial elements and abundant dental material (Extended Data Table 1). These remains primarily come from a single bone bed in the lower part of the archaeological deposits. This concentration, stratigraphic observations made by previous excavators and the anatomical similarity with earlier discoveries strongly suggest that most, if not all, of the hominin remains from the site were accumulated in a rather constrained window of time corresponding to the formation of layer 7. This layer contains the remains of at least five individuals (three adults, one adolescent and one immature individual, around 7.5 years old). The age of the site was redated to  $315 \pm 34$  kyr (as determined by thermoluminescence dating)<sup>3</sup>, consistent with a series of newly established U-series/ESR dates, which places the Irhoud evidence in an entirely new perspective. When compared to the large, robust and prognathic faces of the Neanderthals or older Middle Pleistocene forms, the facial morphologies

of EMH and recent modern humans (RMH) are very distinctive.

The face is relatively short and retracted under the braincase. Facial structures are coronally oriented and the infraorbital area is an ‘inflexion’

type, displaying curvatures along the horizontal, sagittal and coronal profiles. This pattern, which may include some primitive retentions<sup>19</sup>, strongly influences the morphology of the maxilla and zygomatic bone. Our morphometric analysis (Fig. 3 and Methods) clearly distinguishes archaic Middle Pleistocene humans and Neanderthals from RMH. By contrast, all the possible reconstructions of the new facial remains of Irhoud 10 fall well within RMH variation, as does Irhoud 1. Another facial characteristic observed in RMH is the weakness of their brow ridges. Some EMH from Africa and the Levant still have protruding supraorbital structures, but they tend to be dissociated into a medial superciliary arch and a lateral supraorbital arch. Among the Irhoud hominins these structures are rather variable and this variability may be related to sexual dimorphism. Irhoud 1 has protruding

supraorbital structures and the arches are poorly separated. However, in frontal view, the supraorbital buttress tends to form an inverted V above each orbit. On Irhoud 2, the torus is less projecting and a modern pattern can already be seen, with a clear sulcus separating the two arches. On Irhoud 10, the preserved parts do not show projecting supraorbital structures (Fig. 1). The new Irhoud 11 mandible is very large overall (Fig. 2 and Extended Data Table 2). As in some EMH from the Levant or north Africa, it has retained a vertical symphysis, with a mental angle of  $88.8^\circ$  (Extended Data Fig. 1). The mandibular body has a pattern typical of *H. sapiens*: its height strongly decreases from the front to the back. This feature is also present in the immature individual, Irhoud 3. Another modern aspect of Irhoud 11 is the rather narrow section of the mandibular body expressed by the breadth/height index at the level of the mental foramen (Extended Data Fig. 1).

The Irhoud mandibles also show some derived conditions in the mental area (Extended Data Fig. 1). The symphyseal section of Irhoud 11 has a tear-shaped outline quite distinctive of *H. sapiens*. Although the Irhoud mandibles lack a marked mandibular incurvation, the juvenile Irhoud 3 has a central keel between two depressions expanding inferiorly into a thickened triangular eminence. This inverted T-shape, typical of recent *H. sapiens*<sup>20</sup>, is incipient in the adult. Its inferior border is somewhat distended and includes separated tubercles. Notably, this modern pattern is still inconsistently present in Levantine EMH<sup>20</sup>. In some aspects, Irhoud 11 is evocative of the Tabun C2 mandible, but it is much more robust. The Irhoud teeth are generally very large (Extended Data Tables 3, 4).

However, their dental morphology is reminiscent of EMH in several respects. The anterior teeth do not display the expansion observed in non-sapiens Middle Pleistocene hominins and Neanderthals<sup>21</sup> and the post-canine teeth are reduced compared to older hominins. The third maxillary molar (M3) of Irhoud 21 is already smaller than in some EMH. The crown morphology (Extended Data Table 5 and Extended Data Fig. 2) also aligns the Irhoud specimens most closely with

*H. sapiens*, rather than with non-sapiens Middle Pleistocene hominins and Neanderthals. They do not display expanded and protruding first upper molar (M1) hypocones, lower molar middle trigonid crests (especially at the enamel–dentine junction (EDJ)), or a second lower premolar (P4) with a transverse crest, uninterrupted by a longitudinal

fissure. The molars are morphologically complex and similar to the large teeth of African EMH, possessing accessory features such as a cusp 6, cusp 7 and protostylid on the lower molars and cusp 5 on the upper molars. The EDJ analysis demonstrates the retention of a non-Neanderthal primitive pattern of the P4 (Extended Data Fig. 2b). However, derived crown shapes shared with RMH are already seen in the upper and lower molars, grouping Irhoud 11 with EMH from north Africa and the Levant. The lower incisor and canine roots retain a large size, but the shape is already within the range of the modern distribution (Extended Data Fig. 3). Mandibular molar roots are cynodont, that is, modern-human-like. This mandibular root configuration of Irhoud 11 is similar to that observed in EMH from Qafzeh. Finally, Irhoud

3 shows a pattern of eruption and a period of dental development close to recent *H. sapiens*<sup>13</sup>.

Figure 2 | Irhoud 11 mandible (lateral and occlusal views). See Methods for the reconstruction. The bi-condylar breadth of the Irhoud 11 mandible fits the width of the corresponding areas on the Irhoud 2 skull exactly. Scale bar, 20 mm.

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In contrast to their modern facial morphology, the Irhoud crania retain a primitive overall shape of the braincase and endocranium, that is, unlike those of RMH, they are elongated and not globular<sup>10,18,22</sup>. This results in a low outline of the occipital squama, elongated temporal bones and a low convexity of the parietal<sup>11</sup>. However, the frontal squama has a vertical orientation and a marked convexity when compared to archaic Middle Pleistocene specimens. These derived conditions are especially well expressed on Irhoud 2 (ref. 11). A geometric

morphometric analysis (Extended Data Fig. 4) of external vault shape distinguishes Neanderthals and archaic Middle Pleistocene forms with their primitive neurocranial shape from RMH and Upper Palaeolithic humans. With regards to the first principal component (PC)1, Irhoud 1 and 2 are intermediate and group together with specimens such as Laetoli H18 and Qafzeh, as well as Upper Palaeolithic individuals from Mladeč or Zhoukoudian Upper Cave. To some degree all of these specimens retained longer and lower braincase proportions compared to RMH. The morphometric analysis of endocranial shape (Fig. 3b), which is not affected by cranial superstructures, shows a clear separation between *H. erectus* and the Neanderthal/archaic Middle Pleistocene cluster along PC2. The latter have evolved larger neocortices but, in contrast to RMH, without a proportional increase in the cerebellum (Extended Data Fig. 5). EMH and the Irhoud hominins also display elongated endocranial profiles, but are intermediate between *H. erectus* and the cluster of Neanderthals/archaic Middle Pleistocene hominins along PC2. They range in rough agreement with their

geological age along PC1, in a morphological cline ending with the extant globular brain shapes of RMH. Notably, Omo Kibish 2 falls between Irhoud 1 and 2. This similarity continues the question of the contemporaneity of Omo Kibish 1 and 2, two specimens with very different braincase morphologies<sup>23</sup>. The Irhoud fossils currently represent, to our knowledge, the most securely dated evidence of the early phase of *H. sapiens* evolution in Africa, and they do not simply appear as intermediate between African archaic Middle Pleistocene forms and RMH. Even approximately 300 ka ago their facial morphology is almost indistinguishable from that of RMH, corroborating the interpretation of the fragmentary specimen from Florisbad (South Africa) as a primitive

*H. sapiens* tentatively dated to 260 ka (ref. 24). Mandibular and dental morphology, as well as the pattern of dental development also align the Irhoud fossils with EMH. Notably, the endocranial analysis suggests diverging evolutionary trajectories between early *H. sapiens* and African archaic Middle Pleistocene forms. This anatomical evidence and the chronological proximity between these two groups<sup>25</sup> reinforce the hypothesis of a rapid anatomical shift or even, as suggested by some<sup>26</sup>, of a chronological overlap. The Irhoud evidence supports a complex evolutionary history of *H. sapiens* involving the whole African continent<sup>25,27</sup>. Like in the Neanderthal lineage<sup>28</sup>, facial morphology was established early on, and evolution in the last 300 ka primarily affected the braincase. This

occurred together with a series of genetic changes affecting brain connectivity<sup>29</sup>, organization and development<sup>22</sup>. Through accretional changes, the Irhoud morphology is directly evolvable into that of extant humans. Delimiting clear-cut anatomical boundaries for a 'modern' grade within the *H. sapiens* clade thus only depends on gaps in the fossil record<sup>30</sup>.

**18. Which statement about inflammation is incorrect:**

- a. In acute inflammation, the immune system releases chemical messengers to the injured area.
- b. In acute inflammation, the immune system releases white blood cells to the injured area.
- c. Redness, warmth, swelling, and pain are normal reactions to acute inflammation.
- d. Irritants are removed quickly in chronic inflammation.

**ABSTRACT** Inflammation is a biological response of the immune system that can be triggered by a variety of factors, including pathogens, damaged cells and toxic compounds. These factors may induce acute and/or chronic inflammatory responses in the heart, pancreas, liver, kidney, lung, brain, intestinal tract and reproductive system, potentially leading to tissue damage or disease. Both infectious and non-infectious agents and cell damage activate inflammatory cells and trigger inflammatory signaling pathways, most commonly the NF- $\kappa$ B, MAPK, and JAK-STAT pathways. Here, we review inflammatory responses within organs, focusing on the etiology of inflammation, inflammatory response mechanisms, resolution of inflammation, and organ-specific inflammatory responses.

**INTRODUCTION**

Inflammation is the immune system's response to harmful stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation [1], and acts by removing injurious stimuli and initiating the healing process [2]. Inflammation is therefore a defense mechanism that is vital to health [3]. Usually, during acute inflammatory responses, cellular and molecular events and interactions efficiently minimize impending injury or infection. This mitigation process contributes to restoration of tissue homeostasis and resolution of the acute inflammation. However, uncontrolled acute inflammation may become chronic, contributing to a variety of chronic inflammatory diseases [4]. At the tissue level, inflammation is characterized by redness, swelling, heat, pain, and loss of tissue function, which result from local immune, vascular and inflammatory cell responses to infection or injury [5]. Important microcirculatory events that occur during the inflammatory process include vascular permeability changes, leukocyte recruitment and accumulation, and inflammatory mediator release [2, 6].

Various pathogenic factors, such as infection, tissue injury, or cardiac infarction, can induce inflammation by causing tissue damage. The etiologies of inflammation can be infectious or non-infectious (Table 1). In response to tissue injury, the body initiates a chemical signaling cascade that stimulates responses aimed at healing affected tissues. These signals activate leukocyte chemotaxis from the general circulation to sites of damage. These

activated leukocytes produce cytokines that induce inflammatory responses [7].

#### INFLAMMATORY RESPONSE MECHANISMS

The inflammatory response is the coordinate activation of signaling pathways that regulate inflammatory mediator levels in resident tissue cells and inflammatory cells recruited from the blood [8]. Inflammation is a common pathogenesis of many chronic diseases, including cardiovascular and bowel diseases, diabetes, arthritis, and cancer [9]. Although inflammatory response processes depend on the precise nature of the initial stimulus and its location in the body, they all

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share a common mechanism, which can be summarized as follows: 1) cell surface pattern receptors recognize detrimental stimuli; 2) inflammatory pathways are activated; 3) inflammatory markers are released; and 4) inflammatory cells are recruited.

Pattern recognition receptor activation Microbial structures known as pathogen-associated molecular patterns (PAMPs) can trigger the inflammatory response through activation of germline-encoded pattern-recognition receptors (PRRs) expressed in both immune and nonimmune cells [10, 11]. Some PRRs also recognize various endogenous signals activated during tissue or cell damage and are known as danger-associated molecular patterns (DAMPs) [11]. DAMPs are host biomolecules that can initiate and perpetuate a noninfectious inflammatory response [12]. Disrupted cells can also recruit innate inflammatory cells in the absence of pathogens by releasing DAMPs [13]. Classes of PRR families include the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs) [5]. TLRs are a family of highly conserved, mammalian PRRs that participate in the activation of the inflammatory response [14]. More than ten members of the TLR family have been identified, and TLRs are the most well-studied of the known PRRs [15]. Transmission of PAMPs and DAMPs is mediated by myeloid differentiation factor-88 (MyD88) along with TLRs. Signaling through TLRs activates an intracellular signaling cascade [16, 17] that leads to nuclear translocation of transcription factors, such as activator protein-1 (AP-1) and NF- $\kappa$ B or interferon regulatory factor 3 (IRF3) (Figure 1). DAMPs and PAMPs share receptors, such as TLR4, suggesting similarities between infectious and noninfectious inflammatory responses [18, 19].

Activation of inflammatory pathways Inflammatory pathways impact the pathogenesis of a number of chronic diseases, and involve common inflammatory mediators and regulatory pathways. Inflammatory stimuli activate intracellular signaling pathways that then activate production of inflammatory mediators. Primary inflammatory stimuli, including microbial products and cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), mediate inflammation through interaction with the TLRs, IL-1 receptor (IL-1R), IL-6 receptor (IL-6R), and the TNF receptor (TNFR) [20]. Receptor activation triggers important intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF- $\kappa$ B), and Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathways [21–23].

NF- $\kappa$ B pathway The NF- $\kappa$ B transcription factor plays important roles in inflammatory, immune response, survival, and apoptosis processes [24]. The NF- $\kappa$ B family includes five related transcription factors: P50, p52, RelA (p65), RelB, and c-Rel [25, 26]. NF- $\kappa$ B activity

is induced by a range of stimuli, including pathogen-derived substances, intercellular inflammatory cytokines, and many enzymes [27, 28]. Under physiological conditions, I $\kappa$ B proteins present in the cytoplasm inhibit NF- $\kappa$ B [29]. PRRs use similar signal transduction mechanisms to activate I $\kappa$ B kinase (IKK), which is composed of two kinase subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit, such as IKK $\gamma$ . IKK regulates NF- $\kappa$ B pathway activation through I $\kappa$ B phosphorylation [8]. I $\kappa$ B phosphorylation results in its degradation by the proteasome and the subsequent release of NF- $\kappa$ B for nuclear translocation and gene transcription activation [30]. This pathway regulates pro-inflammatory cytokine production and inflammatory cell recruitment, which contribute to the inflammatory response (Figure 2).

MAPK pathway MAPKs are a family of serine/threonine protein kinases that direct cellular responses to a variety of stimuli, including osmotic stress, mitogens, heat shock, and inflammatory cytokines (such as IL-1, TNF- $\alpha$ , and IL6), which regulate cell proliferation, differentiation, cell survival and apoptosis [31, 32]. The mammalian MAPKs include extracellular-signal-regulated kinase ERK1/2, p38 MAP Kinase, and c-Jun N-terminal kinases (JNK) [33]. Each MAPK signaling pathway comprises at least

Table 1: Etiology of inflammation  
 Non-infectious factors  
 Infectious factors  
 Physical: burn, frostbite, physical injury, foreign bodies, trauma, ionizing radiation  
 Chemical: glucose, fatty acids, toxins, alcohol, chemical irritants (including fluoride, nickel and other trace elements)  
 Biological: damaged cells  
 Psychological: excitement  
 Bacteria viruses other microorganisms

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three components: a MAPK, a MAPK kinase (MAPKK), and a MAPK kinase kinase (MAPKKK). MAPKKKs phosphorylate and activate MAPKKs, which in turn phosphorylate and activate MAPKs [33, 34]. ERKs are generally activated by mitogens and differentiation signals,

while inflammatory stimuli and stress activate JNK and p38 [35]. MKK1 and MKK2 activate ERK1/2, MKK4 and MKK7 activate JNK, and MKK3 and MKK6 activate p38. Activation of the MAPKs, including Erk1/2, JNK, leads to phosphorylation and activation of p38 transcription factors

Figure 1: TLR signaling. MyD88-dependent and TRIF-dependent pathways are shown. Signaling through TLRs activates intracellular signaling cascades that lead to nuclear translocation of AP-1 and NF- $\kappa$ B or IRF3, which regulates the inflammatory response.

Figure 2: NF- $\kappa$ B pathway. This pathway is triggered by TLRs and inflammatory cytokines, such as TNF and IL-1, leading to activation of RelA/p50 complexes that regulate expression of inflammatory cytokines. NF- $\kappa$ B signaling requires IKK subunits. which regulate pathway activation through I $\kappa$ B phosphorylation.

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present in the cytoplasm or nucleus, which initiates the inflammatory response [32, 36] (Figure 3).

JAK-STAT pathway The highly conserved JAK-STAT pathway involves diverse cytokines, growth factors, interferons, and related molecules, such as leptin and growth hormone, and is a signaling mechanism through which extracellular factors can control gene expression [37]. Receptor-associated JAKs are activated by ligands and phosphorylate one other, creating docking sites for STATs, which are latent, cytoplasmic transcription factors. Cytoplasmic STATs recruited to these sites undergo phosphorylation and subsequent

dimerization before translocation to the nucleus [38]. Tyrosine phosphorylation is essential for STAT dimerization and DNA binding [39]. Therefore, JAK/STAT signaling allows for the direct translation of an extracellular signal into a transcriptional response. For example, binding of IL-6 family members to plasma membrane receptors activates the JAK-STAT proteins. STAT proteins translocated into the nucleus bind target gene promoter regions to regulate transcription of inflammatory genes (Figure 4) [40]. Dysregulation of NF- $\kappa$ B, MAPK, or JAK-STAT activity is associated with inflammatory, autoimmune, and metabolic diseases, and cancer [41]. Signaling through transcription factors results in secretion of cytokines [42, 43]. Multiple transcription factors regulate a variety of inflammatory genes, such as IL-1, TNF- $\alpha$ , IL-6 [44], colony stimulating factor (CSF), interferons, transforming growth factor (TGF), and chemokines.

**Inflammatory markers** Markers are used in clinical applications to indicate normal versus pathogenic biological processes, and assess responses to therapeutic interventions. Inflammatory markers may be predictive of inflammatory diseases [45–50], and correlate with the causes and consequences of various inflammatory diseases, such as cardiovascular diseases, endothelial dysfunctions, and infection [51, 52]. Stimuli activate inflammatory cells, such as macrophages and adipocytes, and induce production of inflammatory cytokines, such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and inflammatory proteins and enzymes. These molecules can potentially serve as biomarkers for diseases diagnosis, prognosis, and therapeutic decision making [53–57].

**Inflammatory cytokines** Cytokines (Table 2) are predominantly released from immune cells, including monocytes, macrophages, and lymphocytes. Pro- and anti-inflammatory cytokines facilitate and inhibit inflammation, respectively. Inflammatory cytokines are classified as ILs, colony stimulating factors (CSF), IFNs, TNFs, TGFs, and chemokines, and are produced by cells primarily to recruit leukocytes to the site of infection or injury [58]. Cytokines modulate the immune response to infection or inflammation and regulate inflammation itself via a complex network of interactions. However, excessive inflammatory cytokine production can lead to tissue damage, hemodynamic changes, organ failure, and ultimately death [59, 60]. A better understanding of how to regulate cytokine pathways would allow for more accurate identification of agent-mediated inflammation and the treatment of inflammatory diseases [58].

**Inflammatory proteins and enzymes** Inflammatory proteins in the blood, including C-reactive protein (CRP), haptoglobin, serum amyloid A, fibrinogen, and alpha 1-acid glycoprotein [61], help restore homeostasis and reduce microbial growth independently of antibodies during trauma, stress, or infection [62]. Abnormal activation of certain enzymes, including high-mobility group box 1 (HMGB1), superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, play key roles in the development of inflammation-related diseases, such as cardiovascular disease and cancer [63–66]. For example, extracellular HMGB1 effects may be mediated by activation of TLR-coupled signaling pathways [67]. The primary target of extracellular HMGB1 is TLR4 [68], which triggers MyD88-dependent intracellular signaling cascades involved in activation of the NF- $\kappa$ B and MAPK pathways. This leads to release of such inflammatory cytokines as TNF- $\alpha$  and IL-1 $\beta$  [67].

Inflammatory proteins and enzymes have been used as inflammation, infection, and trauma biomarkers in medicine.

Other inflammatory markers Antioxidant defense systems, including antioxidant enzymes, influence oxidative stress. Elevated oxidative stress can induce production of reactive oxygen species (ROS), malondialdehyde (MDA), 8-Hydroxy-2deoxyguanosine (8-OHdG) and isoprostanes [64, 69], each of which can activate various transcription factors, including NF- $\kappa$ B, AP-1, p53, and STAT. Thus, this cascade can increase expression of genes encoding growth factors, inflammatory cytokines, and chemokines [70]. Oxidative stress is associated with the pathogenesis of multiple diseases, such as cardiovascular disease, cancer, diabetes, hypertension, aging, and atherosclerosis. Therefore, oxidative stress products can also be used as markers of the inflammatory response.

Cell types in inflammatory responses The inflammatory response involves a highly coordinated network of many cell types. Activated

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macrophages, monocytes, and other cells mediate local responses to tissue damage and infection. At sites of tissue injury, damaged epithelial and endothelial cells release factors that trigger the inflammatory cascade, along with chemokines and growth factors, which attract neutrophils and monocytes. The first cells attracted to a site of injury are neutrophils, followed by monocytes, lymphocytes (natural killer cells [NK cells], T cells, and B cells), and mast cells [71–73]. Monocytes can differentiate into macrophages and dendritic cells and are recruited via

chemotaxis into damaged tissues. Inflammation-mediated immune cell alterations are associated with many diseases, including asthma, cancer, chronic inflammatory diseases, atherosclerosis, diabetes, and autoimmune and degenerative diseases. Neutrophils, which target microorganisms in the body, can also damage host cells and tissues [74]. Neutrophils are key mediators of the inflammatory response, and program antigen presenting cells to activate T cells and release localized factors to attract monocytes and

Table 2: Summary of cytokines and their functions Cytokine Family Main sources Function

IL-1 $\beta$  IL-1 Macrophages, monocytes Pro-inflammation, proliferation, apoptosis, differentiation

IL-4 IL-4 Th-cells Anti-inflammation, T-cell and B-cell proliferation, B-cell differentiation

IL-6 IL-6 Macrophages, T-cells, adipocyte Pro-inflammation, differentiation, cytokine production

IL-8 CXC Macrophages, epithelial cells, endothelial cells Pro-inflammation, chemotaxis, angiogenesis

IL-10 IL-10 Monocytes, T-cells, B-cells Anti-inflammation, inhibition of the pro-inflammatory cytokines

IL-12 IL-12 Dendritic cells, macrophages, neutrophils Pro-inflammation, cell differentiation, activates NK cell

IL-11 IL-6 Fibroblasts, neurons, epithelial cells Anti-inflammation, differentiation, induces acute phase protein

TNF- $\alpha$  TNF Macrophages, NK cells, CD4+ lymphocytes, adipocyte

Pro-inflammation, cytokine production, cell proliferation, apoptosis, anti-infection

IFN- $\gamma$  INF T-cells, NK cells, NKT cells Pro-inflammation, innate, adaptive immunity anti-viral

GM-CSF IL-4 T-cells, macrophages, fibroblasts Pro-inflammation, macrophage activation, increase neutrophil and monocyte function

TGF- $\beta$  TGF Macrophages, T cells Anti-inflammation, inhibition of pro-inflammatory cytokine production

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dendritic cells [7]. Macrophages are important components of the mononuclear phagocyte system, and are critical in inflammation initiation, maintenance, and resolution [75]. During inflammation, macrophages present antigens, undergo phagocytosis, and modulate the immune response by producing cytokines and growth factors. Mast cells, which reside in connective tissue matrices and on epithelial surfaces, are effector cells that initiate inflammatory responses. Activated mast cells release a variety of inflammatory mediators, including cytokines, chemokines, histamine, proteases, prostaglandins, leukotrienes, and serglycin proteoglycans [76]. Multiple groups have demonstrated that platelets impact inflammatory processes, from atherosclerosis to infection. Platelet interactions with inflammatory cells may mediate pro-inflammatory outcomes. The acute phase response (APR) is the earliest response to infection or injury, and some studies have indicated that platelets induce the APR [77]. After being recruited by inflammatory stimuli, immune cells amplify and sustain the APR by releasing local inflammatory mediators at the site of recruitment. RESOLUTION OF INFLAMMATION

To prevent progression from acute inflammation to persistent, chronic inflammation, the inflammatory response must be suppressed to prevent additional tissue damage. Inflammation resolution is a well-managed process involving the spatially- and temporally-controlled production of mediators, during which chemokine gradients are diluted over time.

Circulating white blood cells eventually no longer sense these gradients and are not recruited to sites of injury. Dysregulation of this process can lead to uncontrolled chronic inflammation [78]. Inflammation resolution processes that rectify tissue homeostasis include reduction or cessation of tissue infiltration by neutrophils and apoptosis of spent neutrophils, counter-regulation of chemokines and cytokines, macrophage transformation from classically to alternatively activated cells, and initiation of healing [79, 80]. Chronic inflammation occurs when acute inflammatory mechanisms fail to eliminate tissue injury [81], and may lead to a host of diseases, such as cardiovascular diseases, atherosclerosis, type 2 diabetes, rheumatoid arthritis, and cancers [82]. Understanding the common mechanisms that orchestrate dysfunction in the various organ systems will allow for development and production of improved targeted therapies.

#### ORGAN-SPECIFIC INFLAMMATORY RESPONSES

Inflammation has long been recognized as a major cause of disease. It is estimated that some 15% of human cancers are associated with chronic infection and inflammation [83]. Acute and chronic inflammation-mediated tissue injury is observed in many organ systems, including the heart, pancreas, liver, kidney, lung, brain, intestinal tract, and reproductive system.

Figure 3: MAPK pathway. This pathway mediates intracellular signaling initiated by extracellular stimuli, such as stress and cytokines. MAPKKs phosphorylate and activate MAPKKs, which in turn phosphorylate and activate MAPKs. The mammalian MAPK family includes Erk1/2, JNK, and p38. In the Erk1/2 pathway, Erk1/2 is activated by MKK1/2, which is activated by Raf. In the JNK pathway, JNK is activated by MKK4/7, which is activated by MEKK1/4, ASK1, and MLK3. In the p38 pathway, p38 is activated by MKK3/6,

which is activated by MLK3, TAK, and DLK. Activated MAPKs phosphorylate various proteins, including transcription factors, resulting in regulation of inflammatory responses. Oncotarget 7210 www.impactjournals.com/oncotarget

Heart Cardiovascular disease, and its underlying pathology, atherosclerosis, is the major cause of death and disability worldwide [84, 85]. By 2030, almost 23.6 million people are projected to die annually from cardiovascular disorders [86, 87]. Inflammatory mediators play key roles in atherosclerosis, from initial leukocyte recruitment through rupture of the atherosclerotic plaque [88–91]. Inflammation is also an early event in cardiac stress. Elevated levels of endothelial adhesion molecules and increased inflammatory cytokine and chemokine production and release are observed in affected cardiac tissues [92]. The innate immune system is the primary cardiac defense against pathogens and tissue damage [93]. Myocardial infarction, which commonly results from coronary atherosclerosis and involves acute loss of many myocardial cells, is the most common cause of cardiac injury [94]. Necrotic cardiac cells initiate an inflammatory cascade to clear dead cells and debris from the infarct [95, 96]. Cell death releases intracellular components that activate innate immune mechanisms to initiate an inflammatory response. Endogenous ligands released following injury are recognized as danger signals by cell surface receptors, and activate inflammation [97, 98]. TLR-mediated pathways trigger post-infarction inflammatory responses by activating NF- $\kappa$ B signaling [98–103]. Chemokines recruit inflammatory leukocytes to the infarct, and cytokines promote leukocyte-endothelial cell adhesions [104, 105]. Moreover, TGF- $\beta$  and IL-10 promote cardiac repair by suppressing inflammation, enhancing myofibroblast phenotypic modulation, and promoting extracellular matrix deposition [106, 107]. Cardiovascular disease is the main cause of death and disability in patients with diabetes mellitus, especially those with type 2 diabetes (T2D), in whom cardiovascular disease occurs 14.6 years earlier on average [108]. About two-thirds of deaths in people with diabetes are due to cardiovascular disease; among these, approximately 40% die from ischemic heart disease, 15% from other forms of heart disease, principally congestive heart failure, and about 10% from stroke [109]. Recent global estimates indicate that over 422 million adults currently live with diabetes, of which over 90% have T2D. Diabetes is a group of metabolic disorders characterized by sustained high blood sugar levels, and is a major global health challenge, both to individuals and their families, and to healthcare systems [110]. Diabetes complications include heart attack, stroke, kidney failure, limb amputation, blindness, and nerve damage. Diabetes results from either impaired insulin production in the pancreas or body cells not responding to produced insulin [111]. Insulin resistance is defined as decreased insulin-stimulated glucose uptake, and is associated with inactivity, obesity, and aging. Pancreatic islet cells respond to insulin resistance by enhancing insulin secretion and cell mass. However, when islet  $\beta$ -cells are unable to compensate for insulin resistance, insulin deficiency develops, followed by T2D [112], which is increasingly being characterized as an inflammatory disease [113, 114]. Elevated circulating levels of acute-phase proteins, including CRP, fibrinogen, serum amyloid A, plasminogen activator inhibitor, and haptoglobin, along with sialic acid, cytokines, and chemokines, have been observed in patients with T2D. Elevated IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and CRP levels are also predictive of T2D. IL-1 receptor antagonist (IL-1RA) is elevated in obesity and prediabetes prior to T2D onset. Excessive nutrient levels, including those of glucose and free fatty acids, promote insulin resistance. T2D also activates the NF- $\kappa$ B, MAPK, and JAK-STAT pathways,

which can each promote tissue inflammation [110, 114, 115]. Metabolic stressors also negatively impact pancreatic islet cells and insulin-sensitive tissues, including adipose tissue, promoting local cytokine and chemokine production and release. At the same time, immune cells, such as mast cells and macrophages, are recruited and contribute to

Figure 4: JAK-STAT pathway. Following IL-6 binding, signal is transduced by a receptor to activate the JAKs, which then activate STATs. STATs are dephosphorylated in the nucleus, leading to activation of downstream cytokines.

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tissue inflammation. Similarly, cytokine and chemokine release from adipose tissues into the circulation promotes further inflammation in other tissues [116].

Pancreas Pancreatitis, caused by pancreatic duct obstruction, trypsinogen gene mutation, or alcoholism, is an inflammatory disease of the pancreas [117]. Acute pancreatitis (AP) incidence ranges from 4–45 per 100,000 patients per year and increases annually by approximately 1.3–4.0% in most developed countries. AP is one of the most common gastrointestinal causes for hospitalization in the US, and chronic pancreatitis (CP) is less common than AP. However, CP patients experience chronic abdominal pain and exocrine and/or endocrine insufficiency, leading to reduced quality of life [118]. Pancreatitis is characterized by acinar cell destruction and activation of inflammatory cells, including macrophages, neutrophils, and granulocytes, which secrete inflammatory cytokines [117, 119]. These cytokines further activate pancreatic stellate cells (PSCs) to promote CP [120]. Pancreatitis development requires various molecular pathways, such as NF- $\kappa$ B, MAPK, and JAK-STAT, which play critical roles in inflammatory cell activation during pancreatitis [117]. Pancreatic cancer (PC) remains one of the most lethal of malignancies and a major health burden [121], and is the fourth most common cause of death from cancer in the US [118]. There is a strong link between antecedent CP and PC [122]. CP leads to fibrosis, which is a common pathological feature and major risk factor for PC [123]. Pancreatic cancer results from dysregulation of oncogenes and tumor suppressor genes, as well as growth factors and their receptors, including epidermal growth factors, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and many cytokines, such as TGF- $\beta$ , IL-1, IL-6, TNF- $\alpha$ , and IL-8, which modulate pathways involved in growth and differentiation [124, 125]. Shi, et al. has showed that VEGF is upregulated by low extracellular PH (acidosis), which occurs frequently around necrotic regions in tumors, and that acidosis activates IL-8 [126]. VEGF and IL-8 are important angiogenic factors in PC [126], and acidosis-promoted upregulation of these genes can be mediated through NF- $\kappa$ B and AP-1 transactivation and cooperation [127].

Liver Inflammation in the liver protects this organ from infection and injury, but excessive inflammation may lead to extensive loss of hepatocytes, ischemia-reperfusion injury, metabolic alterations, and eventually permanent hepatic damage [128]. Inflammation can destroy hepatic parenchymal cells, increasing the risk of chronic liver diseases, such as non-alcoholic fatty liver disease (NAFLD) or viral hepatitis. Chronic liver diseases are a leading cause of morbidity and mortality in the US [129].

The liver is the largest solid organ in the body [130], and is a target of both infectious and non-infectious inflammatory pathologies. Infectious inflammation of the liver is mainly caused by microorganisms, such as bacterial products, hepatitis B virus (HBV), or hepatitis C virus (HCV) [131, 132]. Sterile inflammation (SI) is also important in the pathology of many liver diseases, such as alcoholic or nonalcoholic steatohepatitis, drug-induced liver

injury, and ischemia/reperfusion [133–135]. In SI, endogenous DAMPs are released to injured tissues and activate immune cells [136]. While pathogen-driven inflammation and SI differ, they share several functional characteristics. Many receptors and pathways can be activated by both PAMPs and DAMPs [137]. TLR4, for example, can be activated by bacterial LPS and cellular HMGB1. Because of the liver's unique vascular supply, PAMPs of intestinal origin and DAMPs from hepatocytes both contribute to inflammation in a variety of diseases. For example, PRR activation by DAMPs and PAMPs can induce production of pro-inflammatory cytokines and immune cell localization to sites of injury. Recognition of DAMPs and PAMPs results in assembly of the inflammasome, a cytosolic protein complex that activates the serine protease caspase-1, followed by activation and secretion of IL-1 $\beta$  and other cytokines. At the same time, Kupffer cell activation and inflammatory cell recruitment leads to production of cytokines and chemokines that promote long-term inflammation, hepatocyte damage, and/or cholestasis [138].

**Lung** Lung inflammatory diseases involve complex interactions among and between structural and immune cells [139]. Lung inflammation results predominantly from tissue exposure to bacterial and viral pathogens, and/or environmental pollutants. Excessive acute inflammation and subsequent lung injury can cause pulmonary fibrosis and impair gas exchange. Unresolved lung injury and chronic inflammation are frequently observed in acute respiratory distress syndrome, cystic fibrosis, chronic obstructive pulmonary disease (COPD), and asthma [140–142]. Approximately 90% of COPD cases are associated with cigarette smoking-induced inflammation in small airways and lung parenchyma [143].

Cigarette smoking is a major risk factor for COPD, which involves both systemic and pulmonary inflammation. Long-term smoking can cause macrophage, neutrophil, and activated T lymphocyte infiltration into airways, and promote production of chemokines, oxygen radicals, proteases, and cytokines, including that of TNF- $\alpha$ , IL-6 and IL-8, in the lung [144]. **Kidney** Kidney inflammation contributes to progressive renal injury, which may lead to glomerulonephritis, end-stage renal disease, or acute or chronic kidney disease (CKD)

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[145–147]. Approximately 10–12% of the population suffers from CKD, and some 50% of elderly patients show signs of kidney dysfunction, which is associated with high morbidity and mortality [52]. Kidney inflammation is most commonly induced by infection, ischemia/reperfusion, in situ immune complex formation/deposition, or complement pathway dysregulation [145]. CKD and acute kidney injury (AKI) are the most severe types of kidney disease [148]. Interstitial inflammation and tubular injury are commonly observed in acute and chronic kidney injury cases. Renal tubular epithelial cells are likely important promoters of kidney inflammation, secreting a variety of inflammatory cytokines in response to both immune and non-immune factors, and leukocyte infiltration depends on the local presence of these cytokines [146]. Stimuli that can induce kidney injury activate transcription factors (NF- $\kappa$ B or MAPK). These stimuli include cytokines, growth factors, DAMPs, and PAMPs, TLRs, Nod-like receptors (NLRs), and metabolic (high glucose, advanced glycosylation end products) and immune mediators [147]. **Intestinal tract** Acute and chronic inflammatory diseases of the intestine can cause various health issues, and decrease patient quality of life worldwide [149, 150]. The complex, polygenetic inflammatory bowel diseases (IBDs) are characterized by an excessive inflammatory response to gut lumen microbial flora [151]. IBDs mainly include ulcerative colitis (UC) and Crohn disease (CD), but also noninfectious inflammation of the bowel [152, 153]. Idiopathic

IBDs, such as CD and UC, are caused by cytokine-driven, non-infectious inflammation of the gut. For example, CD is associated with excessive IFN- $\gamma$ /IL-17 and IL-12/IL-23 production, while UC is associated with excess IL-13 [153]. Thus, IBD appears to be the result of a dysfunctional interaction between gut bacteria and the mucosal immune system [154]. A key process in the immune system's response to microbes is the recognition of microbial agents via PRRs, including TLRs and nucleotide-binding oligomerization domain containing NLRs, which sense evolutionarily conserved PAMPs [155]. Upon PAMP detection, PRRs activate intracellular signaling pathways that induce production of cytokines and chemokines to promote host resistance to infection. TLR (mainly TLR4) signaling induces NF- $\kappa$ B and MAPK transcription. At the same time, NLRs are also activated through ligand detection [154], in turn activating caspase-1, followed by activation and secretion of IL-1 $\beta$ , interleukin-18 and other cytokines [154].

**Reproductive system** The hallmarks of inflammation are observed during many normal reproductive processes, including menstruation, ovulation, implantation, and parturition [156]. Injury and healing caused by menstruation, ovulation, and parturition trigger the inflammatory cascade. However, initiation and maintenance of inflammatory processes are also important components of many reproductive tract diseases. Damaged tissues locally release inflammatory interleukins, growth factors, cytokines, and prostaglandins, which activate signaling pathways and recruit immune cells (e.g. neutrophils and macrophages) to the site of injury. This process synergistically controls tissue remodeling and repair, but can also induce inflammatory diseases [7]. Inflammatory cytokines, including IL-6, are the primary mediators of inflammation-related reproductive tract diseases, and act via signal transduction pathways such as the MAPK pathway [157, 158]. **Brain Inflammation** Inflammatory responses occur in the brain in many central nervous system (CNS) diseases, including autoimmune diseases, neurodegenerative diseases like Alzheimer's (AD) and Parkinson's disease (PD), and epilepsy. Inflammatory responses in the brain can enhance neuronal excitability, injure cells, and increase blood-brain barrier permeability to various molecules [159– 161]. Inflammation-associated CNS diseases result from activation of the brain's resident immune cells and microglia, which produce pro-inflammatory markers [162]. These inflammation processes also involve both the innate and adaptive immune systems and resemble immune responses to systemic infection. Cytokines and TLRs are major inflammatory mediators in the transition between innate and adaptive. Inflammatory responses in the CNS may also be triggered by endogenous ligands recognized by TLRs. DAMPs, such as heat-shock proteins and extracellular matrix degradation molecules, entering the brain through a damaged blood-brain barrier may initiate inflammatory responses. The CNS inflammatory response is strong in reaction to both infectious agents and brain injury, such as tissue damage observed following ischemic, traumatic, or excitotoxic brain injury, or seizure [160, 163, 164].

**CONCLUSIONS**  
Inflammation is frequently a key element in the pathological progression of organ disease. Three main pathways, NF- $\kappa$ B, MAPK, and JAK-STAT, play major roles in inflammation, and dysregulation of one or more of these pathways may lead to inflammation-associated disease. A better understanding of inflammatory response pathways and molecular mechanisms will undoubtedly contribute to improved prevention and treatment of inflammatory diseases.

19. Emotional stress can cause changes in the gut epithelial barrier which can manifest in gingival changes.

- a. True
- b. False

and Degraded the Colonic Mucus Barrier. As increased inflammation is not the reason behind the effect of chronic stress, we attempted to find

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the underlying mechanism. Gut microbiota has emerged as a principal factor in the development and function of the immune system, and research has inextricably linked the gut microbiome to IBD and depression (27–29). However, how chronic stress influences the composition of gut microbiota in normal and DSS-treated mice, and the causality of microbial ecology and the colitis-promoting effect, are poorly understood. We first found a dramatic increase of cultured bacteria in the MLN in the DSS+stress group (Fig. 4A). To further assess the composition of gut microbiota, we performed 16S rRNA gene sequence analysis of the gut microbiota. Principal coordinates analysis (PCoA) revealed a markedly distinct colon microbial landscape in mice in the normal, stress, DSS, and DSS+stress groups (Fig. 4B).

Unweighted pair group method with arithmetic mean (UPGMA) also confirmed the distinct microbial communities among the four groups (Fig. 4C). At the phylum and genus levels, the composition of microbiota all dramatically changed in stress- or DSS-treated mice (Fig. 4D and E). Relative to normal mice, stressed mice harbored increased levels of inflammation-promoting operational taxonomic units (OTUs) related to *Helicobacter*, *Peptostreptococcaceae*, *Streptococcus*, and *Enterococcus faecalis*, but decreased levels of *Rikenella*, *Roseburia*, and *Lachnospiraceae*. In DSS-treated mice, these inflammation-promoting OTUs also bloomed, whereas DSS+stress mice showed a dramatic further increase, especially of *Helicobacter* and *Streptococcus* (Fig. 4F and G).

Fig. 1. Chronic stress accelerated DSS-induced colitis. (A) Body weight of mice in normal and stress groups before DSS and immobility time of mice after 1 mo stress in TST and FST using an EthoVision XT 11.5 system (n = 10). (B) Representative colon pictures and colon length, colon weight, bleeding score, and body weight (n = 9–10). (C) Representative H&E staining, Ki-67 staining, pathological score, and mean positive Ki-67 staining cells counted in 20 crypts (n = 4–5; \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001). (Scale bars: H&E stain, 100 μm; Ki-67 stain, 50 μm.)

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Gut microflora in the intestinal lumen are in close contact with mucin and antimicrobial peptides (Amps) and are profoundly regulated by them. The mucus layer and Amps act together as the first line of defense against commensal microbes and invading pathogens (30). We then rationalized that the integrity of this critical barrier could be compromised by chronic stress. Quantitative PCR results showed a significant decrease in mRNA of Mucin-2 (MUC2), the building block of colonic mucus, and Cdx2, a positive regulator of MUC2 expression, in all stress-treated groups, suggesting a weakened ability for mucus production in stressed mice (Fig. 5A). However, we found a clear increase in mRNA of Klf3 and Tff3, factors involved in barrier function, in the DSS group, and a further increase in the

DSS+stress group (Fig. 5A). These increases could be compensatory responses of the host to offset the mucosal damage induced by DSS and even stress. To further ascertain the effect of stress on mucus, we performed Alcian blue and periodic acid–Schiff (PAS) staining in each mouse. In agreement with the changes in transcripts, results revealed a thinner mucus thickness and a decreased goblet cell number in stressed mice compared with nonstressed mice (Fig. 5 B–D). We also examined the production of Amps, and results showed a significant decrease of lysozyme mRNA expression in all stress- and DSS-treated groups. For cathelicidin Amp (CAMP), however, only the DSS+stress group showed greatly increased expression (Fig. 5E). All these results indicate that mice exposed to chronic stress experience deficiency of mucus layer and lysozymes and changed composition of gut microbiota despite no overt signs of disease, but develop robust colitis after DSS treatment. Gut Microbiota Is Responsible for the Susceptibility of DSS Colitis to Chronic Stress. To probe if the changed gut microbiota triggered the deleterious effect of stress, we took advantage of the transmissible nature of the gut microbiota (15) to determine whether susceptibility to DSS could be enhanced in normal mice by cohousing them with stressed mice. PCoA revealed an equilibrated colon microbial landscape in cohoused mice (Fig. 6A). The abundance of some inflammation-promoting OTUs related to *Streptococcus* and *E. faecalis* were increased in the DSS group after cohousing (Fig. 6B). The composition of microbiota at the phyla and genus levels after cohousing are shown in Fig. S5. Interestingly, even though the weight of mice in the separately housed stress group was significantly lower than in the normal separately housed group, no appreciable differences were seen in mouse weights between normal cohoused and stressed cohoused groups (Fig. 6C). We also observed significant decrease in colon length and body weight and significant increase of bleeding score (Fig. 6D) in DSS cohoused mice compared with DSS separately housed mice, indicating that the transferred gut microbiota from stressed mice to nonstressed mice by cohousing contributes to more severe colitis. Consistent with these findings, the effect of stress on the immune system was compromised by cohousing, as differences in thymus coefficient, MLN coefficient, WBC count, and proportion of MLN-associated immune cells (Fig. 6 D–F) disappeared between cohoused groups.

To further confirm the effect of gut microbiota, we treated mice with antibiotic agents according to a previous study (31). After antibiotic treatment, inflammation-promoting OTUs related to *Helicobacter*, *Peptostreptococcaceae*, *Streptococcus*, and *E. faecalis*

Fig. 2. Chronic stress disturbed the immune system. (A) Flow cytometric analysis of B cells (CD45+, CD19+), NEUs (CD45+, Ly6G+), and ly6chi macrophages in colonic lamina propria (n = 4–5). (B) Representative F4/80 staining images and mean positive cells counted in five high-power fields (HFPs; n = 4–5). (Scale bar: 20  $\mu$ m.) (C) Thymus coefficient, MLN coefficient, and spleen coefficient (n = 9–10). Thymus/MLN/spleen coefficient is calculated as thymus/MLN/spleen weight/ body weight  $\times$  100. (D) WBC count in peripheral blood (n = 4–5). (E) Flow cytometric analysis of B (CD45+, CD19+), CD4+ T (CD45+, CD4+), CD8+ T (CD45+, CD8+), and NEU (CD45+, Ly6G+) cells in MLN (n = 4–5). (F) RT-PCR and ELISA results of IL-6 in colon (n = 5). (G) Western blot results of p-STAT3 in colon (n = 5; \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001).

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were all not detectable (Fig. 6G). Results showed that antibiotic treatment blocked the decrease in body weight induced by stress (Fig. 6

Handl) and partially inhibited the decrease in body weight induced by DSS (Fig. 6I). Remarkably, stress could no longer sensitize mice to DSS colitis in the antibiotic-treated condition and could no longer increase the circulating WBC count (Fig. 6I). Together, these findings reveal the interesting concept that stress-induced microbiota dysbiosis is responsible for susceptibility to DSS-induced colitis.

**Discussion** This study shows that chronic stress changed the microbiota and increased the susceptibility of mice to DSS-induced colitis. Our observation that ablation of the inflammatory cytokine IL-6 did not terminate stress sensitization to DSS colitis suggests that the hyperinflammatory response is not the real culprit. In contrast, the disappearance of differences between stressed and nonstressed groups when the gut microbial landscape was equilibrated by cohousing or microbiota was abolished by antibiotic treatment, unequivocally showing that gut microbiota are responsible for the deleterious effects of stress. Colitis is an immune-related disease, and chronic stress is well known to affect the immune system. Mucosal inflammation was triggered in DSS-induced colitis, with outnumbered infiltration of B cells, NEUs, and macrophages in colonic lamina propria, and significant destruction of the crypt architecture. Colitis was greatly accelerated in chronic stress-treated groups. Despite the inflamed colon, systemic immunity also participated in colitis. The relationships between the lymphatic system and immune response/ inflammation are undeniable. Studies have reported that lymphangitis, compromised lymph drainage and lymphatic pumping, bacterial infiltration, and lymph node infection are likely players in inflammatory disorders and IBD (32–34). We found a decreased MLN coefficient and increased bacterial infiltration in MLN of mice in the DSS+stress group, reflecting more severe colitis in this group. We also discovered compromised thymus in the DSS+stress group, as the thymus coefficient was significantly reduced. All these indicated a colon submucosal hyperinflammation and systemic immune inhibition induced by stress, which contribute to the development of colitis. Multiple signals are involved in the coordination of the immune system, serving to activate and attenuate its responses to attack (35). IL-6 is released upon stimulation of inflammatory cells to activate signaling pathways such as STAT3, and is up-regulated in colitis. Neutralization of IL-6 with antibody showed beneficial effects on colitis (25,36), even though reports have also found genetic deletion of IL-6–promoted colitis (37). We found an increase of IL-6 in the DSS+stress group, and deletion of IL-6 failed to mitigate the effect of stress, suggesting that the hyperinflammatory responses may contribute to the detrimental effect of stress but not to the underlying mechanism behind stress. Despite the driving inflammatory mechanisms, we also found a remarkable decrease of IL-10 mRNA not only in DSS-treated groups but also in the stress only-treated group. This decrease in IL-10 would also contribute to the effect of stress, as IL-10 deficiency manifests in severe intestinal inflammation (38,39), and deletion of IL-10 in animals is one of the oldest colitis models used (40). Immune system can be triggered by gut microbiota (41), and the microbiome of patients with IBD is altered, with cause-and-effect relationships with disease. Numerous studies have shown that colitis can be profoundly affected by transmissible microbial communities that arise from diet changes or host genetic defects (12–15). Here, we found a distinct colon microbial landscape in each group, indicating a dramatically changed composition of microbiota after stress or DSS treatment. Notably, inflammation-promoting OTUs related to *Helicobacter*, *Peptostreptococcaceae*, *Streptococcus*, and *E. faecalis* were increased in the stress-treated group. These findings fit well with evidence that *Helicobacter* is

Fig. 3. Genetic deletion of IL-6 failed to terminate the effect of chronic stress. (A) Body weight before DSS and body weight at the time of euthanasia (n = 5). (B) Colon length, bleeding score, and MLN coefficient (n = 5). (C) Flow cytometric analysis of CD8+ T, NEU, and NK cells in MLN (n = 5). (D) Thymus coefficient (n = 5). (E) Flow cytometric analysis of B and CD4+ T cells in MLN (n = 5). (F) WBC counts in the peripheral blood (n = 5). (G) Flow cytometric analysis of B, NEU, CD4+ T, and CD8+ T cells and ly6chi macrophages in colon (n = 5; \*P < 0.05 and \*\*P < 0.01).

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linked to IBD (42), as are Peptostreptococcaceae (43), Streptococcus (44), and *E. faecalis* (45). Lachnospiraceae, which has shown protection against colitis (46), were significantly decreased after stress. MUC2, the major component of the mucus in the colon, and Amps, the endogenous antibiotics with antimicrobial activities, are expressed in the intestinal lining in close contact with the gut microflora, keeping the majority of gut bacteria away from epithelial cells and also regulating the intestinal microbial habitat (47, 48). Here we observed a decrease in MUC2 mRNA expres

sion and goblet cell numbers and a thinner mucus layer in stresstreated groups. Mucolytic bacteria like Akkermansia were

increasedafterstressorDSS treatment, which may also contribute to the thinner mucus layer.

However, the relative abundance of Akkermansia in the DSS+stress group showed no

increase, and reasons for this might be the lack of mucin, as Akkermansia grow

only on mucin O-glycans as a sole polysaccharides source (10). The underlying mechanisms still

need further study. The decrease in mucus layer induced by chronic stress might facilitate

the development of colitis. It has been reported that MUC2-deficient 129SV

Fig. 4. Chronic stress changed composition of gut microbiota and degraded the colonic

mucus barrier. (A) Representative images and numbers of bacterial colonies in MLN (n = 4;

\*P < 0.05). (B) PCoA (n = 4). (C) UPGMA method (n = 4). (D) Composition of microbiota at

phylum level (n = 4). (E) Composition of microbiota at genus level (n = 4). (F) Bacterial

species from colon content that had the greatest increase in abundance between different

groups and their relative abundance. OTU#120 is related to Helicobacter, OTU#76 to

Peptostreptococcaceae, OTU#41 to Streptococcus, and OTU#38 to *E. faecalis* (n = 4). (G)

Bacterial species from colon content that had the greatest decrease in abundance between

different groups and their relative abundance (\*P < 0.05 and \*\*\*P < 0.001 vs. normal group;

###P < 0.001 vs. DSS group). OTU#23 is related to Rikenella, OTU#24 to Rikenella,

OTU#66 to Roseburia, and OTU#69 to Lachnospiraceae bacterium 6-1 (n = 4).

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mice spontaneously developed colitis (49) even though it is not always the case in C57BL/6J

mice (50–52). However, in C57BL/6J mice,

MUC2 deficiency was associated with low levels of subclinical chronic inflammation (53), and

MUC2 protected against colitis (54). Amps, secreted by goblet cells, intestinal epithelial

cells, Paneth cells, or immune cells, are also important players in IBD (47). It was reported

that colonic MUC2 mucin regulates the expression and antimicrobial activity of  $\beta$ -defensin (55),

and that MUC2 mucin contributes to the

synthesis of CAMP (56). We found significantly decreased expression of lysozyme in stress- and

DSS-treated groups, but an increased expression of CAMP in the DSS+stress group. A

previous study showed that lysozyme might increase mucin gene expression and promote

colonic barrier integrity (57). It was also reported that the expression of Amps is increased in colitis, which represents a self-defense response (47). Reduced expression of MUC2 and lysozyme might contribute to shape the microbial composition of the stress condition. However, the modulation of gut microbiota is remarkably complex and poorly understood, and how chronic stress modulates gut microbiota still needs further study. Of note is that, in the case of cohousing, when the gut microbial habitat is shared between stress and nonstress groups, the increased severity of colitis induced by stress was abrogated, as the severity of DSS colitis was indistinguishable between stressed and nonstressed mice. Microbiota transfer endowed the normal mice with the stress-treated mice's response to DSS, suggesting that the detrimental effect of stress is attributed to the disturbed gut microbiome. Furthermore, the immune system was influenced by microbiota, as differences in thymus coefficient, lymph node coefficient, and infiltrated immune cells disappeared between the cohoused groups. The important role of gut microbiota was further reinforced by antibiotic treatment, as the increased susceptibility to colitis of stressed mice was also abolished after antibiotic treatment. It should be noted that antibiotic treatment only partially blocked the decreased body weight induced by DSS without affecting the colon length or bleeding score. This does not contradict the findings of previous studies because, even though some colitis models show improvement under germ-free conditions or antibiotic treatment, the reports in a DSS model conflict with reports of improvement or promotion (58, 59). However, the reasons for these discrepancies are still unknown. Collectively, the combined effect of cohousing and antibiotic treatment on stress

provides strong evidence pointing to the disturbed gut microbiome as the real mechanism behind the effect of stress. Taken together, our results reveal that chronic stress disturbs gut microbiota, triggering an immune system response, and then facilitates DSS-induced colitis (Fig. 7). This study adds to our understanding of interactions between microbiota and host and provides the basis for future clinical studies for microbiota manipulation and transplantation, and for development of new therapeutic strategies for depression or IBD. Fig. 5. Chronic stress degraded the colonic mucus barrier. (A) RT-PCR results of MUC2, Cdx2, Klf3, and Tff3 (n = 5). (B) Representative images of Alcian blue-stained inner mucus layer. (Scale bar: 20  $\mu$ m.) (C) Representative PAS-stained goblet cell pictures. (Scale bar: 20  $\mu$ m.) (D) Quantification of inner mucus layer thickness and mean PAS+ goblet cells in the colon (n = 4). (E) RT-PCR results of lysozyme and CAMP (n = 5; \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001).

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Fig. 6. Gut microbiota is responsible for the susceptibility of DSS colitis to chronic stress. (A) PCoA of each mouse (n = 4). (B) Bacterial species from colon content that had the greatest increase in abundance between different groups and their relative abundance. OTU#120 is related to *Helicobacter*, OTU#76 to *Peptostreptococcaceae*, OTU#41 to *Streptococcus*, and OTU#38 to *E. faecalis* (n = 4). (C) Body weight before DSS. (D) Body weight at the time of euthanasia, colon length, bleeding score, and WBC count in peripheral blood. (E) MLN and thymus coefficient. (F) Flow cytometric analysis of B cells, CD4+ T cells, NEUs, and CD8+ T cells in MLN (n = 5–6). (G) Bacterial species from colon content in the DSS+stress group in H<sub>2</sub>O and antibiotic-treated conditions (n = 4). (H) Body weight before DSS (n = 6). (I) Body weight at the time of euthanasia, bleeding score, and WBC count in the peripheral blood (n = 6; \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001).

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## IMMUNOLOGY AND INFLAMMATION

**Materials and Methods Mice.** Male C57BL/6 mice were used for all studies. IL-6  $-/-$  mice on C57BL/6 background were purchased from the Model Animal Research Center of Nanjing University, and age-matched C57BL/6 mice were used as WT control. Mice were housed in a room with a 12-h/12-h light/dark cycle and habituated in the room for 3 d before experiments. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (60), with the approval of Center for New Drug Safety Evaluation and Research, China Pharmaceutical University.

**Chronic Restraint Stress.** Mice were randomly assigned to home cage control conditions or chronic restraint stress. The restraint-stress procedure was performed based on previous studies (2, 22). In brief, mice were restrained in well-ventilated conical-bottom centrifuge tubes (50 mL; Corning) for 3 h daily during the procedure, not allowing forward and backward movement. For cohousing experiments, equal numbers of stressed mice and nonstressed mice were housed in the same cage during the procedures.

**Behavior Tests.** Mice behavior was tested by TST and FST by using an EthoVision XT11.5 system. For TST, mice were individually suspended by the distal portion of their tails with adhesive tape for a period of 6 min (30 cm from the floor) in a visually isolated area. For FST, each mouse was placed in a cylinder container (diameter, 28 cm; height, 33 cm) containing 20 cm of water at  $25 \pm 1$  °C. Water was replaced between every trial. Following swim sessions, mice were removed from the cylinder, dried with a towel, and returned to their home cages. Activity monitor software was used to record and quantify the immobility time in both tests.

**Colitis Induction with DSS.** Mice were given 2.5% DSS (36–50 kDa; MP Biomedicals) in drinking water for 7 d to induce colitis and killed on day 8. The length of whole colon was measured, and the weight was recorded after opening longitudinally and flushing with PBS solution. The colon was carefully examined, and the bleeding score was recorded. Half of the colon was used for flow cytometry analysis, and half of the colon was divided into three sections (proximal, middle, and distal). The proximal and distal colon sections were fixed flat on filter paper in 4% phosphate-buffered formaldehyde for histological analyses. The middle colon was then divided into three sections and was snap-frozen for subsequent molecular analyses.

**Histology and Immunohistochemistry.** Proximal and distal colon tissues were fixed in 4% phosphate-buffered formaldehyde solution for 24 h and embedded in paraffin. Sections of 5  $\mu$ m were stained with H&E. Inflammation and tissue damage of each colon was scored based on the degree of epithelial damage and inflammatory infiltrate in the mucosa, submucosa, and muscularis/serosa as previously described (61). Each of the four scores was multiplied by 1 if the change was focal, 2 if it was patchy, and 3 if it was diffuse. The four individual scores per colon were added, resulting in a total scoring range of 0–36 per mouse. For immunohistochemistry, distal colon tissues were cut to 5- $\mu$ m sections. After dewaxing and rehydration, the sections were soaked in sodium citrate buffer for heat-induced epitope retrieval, and incubated with 10% goat serum for 1 h to block the nonspecific binding sites. Then, sections were incubated with anti-Ki-67 antibody (1:200; Abcam) and anti-F4/80 antibody (1:100; Abcam) overnight at 4 °C, followed by incubation with HRP secondary antibodies for 1 h. The sections were

developed by using a diaminobenzidine substrate kit (TIANGEN) and counterstained with hematoxylin. Images were obtained with an Olympus BX41 microscope.

**Cell Isolation and Flow Cytometry.** Isolation of colonic lamina propria cells was performed following a previously established method (62). In brief, luminal content, extraintestinal fat tissue, and blood vessels were removed, and colons were then cut into 0.5-cm pieces. Colon pieces were first incubated with HBSS (without Ca<sup>2+</sup> and Mg<sup>2+</sup>) containing 5% FBS, 2 mM EDTA, and 1 mM DTT to remove epithelial cells and mucus, and then digested in PBS solution containing 5% FBS, 1 mg/mL collagenase VIII (Sigma), and 0.1 mg/mL DNase I (Roche). Digested cell suspension was then washed with PBS solution and filtered with a 45- $\mu$ m cell strainer. Antibodies used for colonic lamina propria staining included CD45 (FITC), CD19 (PECY5), CD11b (PE), Ly6G (PECY7), CD4 (APC), CD8 (PECY7), F4/80 (FITC), CX3CR1 (BV510), and Ly6C (APC). MLN homogenate and spleen homogenate after lysis of red blood cells were also washed with PBS solution and filtered through a 45- $\mu$ m cell strainer to obtain cell suspension. Antibodies used for spleen and MLN staining included CD45 (FITC), CD19 (PECY5), CD4 (APC), CD8 (PECY7), CD11b (PE), Ly6G (PECY7), and CD49 (APC). Cells were analyzed with MACSQuant Analyzer 10 (Miltenyi Biotec). Flow cytometry analysis was done with FlowJo software.

**Real-Time PCR Analysis.** Total RNA from colon tissue was extracted by using TRIzol (Invitrogen) and reverse transcribed into cDNA by using a cDNA synthesis kit (Takara). Quantitative PCR was done with a Step One Plus RealTime PCR system (Applied Biosystems) with gene-specific primers. Expression data were normalized to  $\beta$ -actin mRNA expression.

**Inflammatory Mediator Measurement.** Colon tissues were weighed and homogenized by using a tissue mixer (PRO Scientific) with three volumes of PBS solution. The tissue samples were then centrifuged at 15,000  $\times$  g for 10 min. Tissue supernatants were collected for the assay. TNF- $\alpha$  and IL-6 concentrations were measured by ELISA (Dakewe Biotech). NO concentration was determined by Griess reagent (Beyotime Biotechnology).

**Western Blotting.** Colon tissues were homogenized with lysis buffer and centrifuged. Protein samples were boiled for 5 min, electrophoresed in 10% SDS polyacrylamide gel, and transferred onto PVDF membranes (Millipore). The blots were blocked with 5% skim milk in Tris-buffered saline solution– Tween 0.1% for 1 h at room temperature and probed with primary antibodies at the appropriate dilutions overnight at 4 °C. The blots were washed and incubated for 1 h at room temperature with the HRP-conjugated secondary antibody, then developed with enhanced chemiluminescence (Millipore). The densitometry of protein bands was quantified by using ImageJ software (National Institutes of Health).

**Immunostaining of Mucins and Goblet Cells.** Mucus immunostaining was performed according to a previous study (12). Briefly, mice colon containing fecal material was fixed in methanol-Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid). Tissues were embedded in paraffin in a vertical orientation and cut into 5- $\mu$ m sections. Tissue sections were dewaxed, hydrated, and stained with Alcian blue/Nuclear Fast Red. Thickness of inner mucus was measured in 10 different areas of one section and at least 10 sections of each mouse. For immunostaining of goblet cells, tissue sections were dewaxed, hydrated, and stained with PAS/hematoxylin. PAS+ goblet cells were counted in five different areas of the section and at least 10 sections of each mouse. Measurement and observation were performed with an Olympus BX41 microscope.

**Bacterial Incubation of MLNs.** MLNs were aseptically removed, weighed, and homogenized in PBS solution. The homogenates (100  $\mu$ L) were plated onto Luria–Bertani agar and incubated at 37 °C under aerobic conditions for 24 h, and then the colonies were counted. **Compositional Analysis of the Gut Microbiota by Pyrosequencing and Data Analysis.** Colon content homogenates in PBS solution were immediately frozen (–80 °C) and stored until further processing. Next-generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ. In brief, 30–50 ng DNA was used to generate amplicons by using a MetaVx Library Preparation kit (GENEWIZ). The QIIME data analysis package was used for 16S rRNA data analysis. Sequences were

Fig. 7. A proposed model illustrating influences of chronic stress on colitis. Chronic stress disturbs gut microbiota and impairs the mucus layer, which then triggers the immune system and facilitates colitis.

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grouped into OTUs using the clustering program VSEARCH (1.9.6) against the Silva 119 database preclustered at 97% sequence identity.  $\beta$ -Diversity was calculated by using weighted and unweighted UniFrac, and PCoA was performed. A UPGMA tree from the  $\beta$ -diversity distance matrix was built.

**Antibiotic Treatment.** For antibiotic treatment, broad-spectrum antibiotic agents including ampicillin (1g/L), neomycin (1g/L), and metronidazole (0.5g/L) were administered in drinking water during the time of intervention (31).

**Statistical Analysis.** Data are presented as mean  $\pm$  SEM. Statistical significance was determined by Student's t test between two groups and two-way ANOVA in groups of more than two.  $P < 0.05$  was considered statistically significant.

**Chronic stress and intestinal permeability: Lubiprostone regulates glucocorticoid receptor-mediated changes in colon epithelial tight junction proteins, barrier function, and visceral pain in the rodent and human**

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Abbreviations: ChIP, chromatin immunoprecipitation; CIC-2, chloride channel type 2; CRD, colorectal distention; EMG, electromyography; FITC, fluorescein isothiocyanate; FKBP5, FK506 binding protein 5; GR, glucocorticoid receptor; GRE, glucocorticoid response elements; Hsp, heat-shock protein; IBS, irritable bowel syndrome; Lub, lubiprostone; MR, mineralocorticoid receptor; TEER, transepithelial electrical resistance; VMR, visceral motor response; WA, water avoidance; ZO-1, zonula occludens-1.

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**Abstract Background:** Chronic psychological stress is associated with increased intestinal epithelial permeability and visceral hyperalgesia. Lubiprostone, an agonist for chloride channel-2, promotes secretion and accelerates restoration of injury-induced epithelial barrier dysfunction. The mechanisms underlying how lubiprostone regulates colon epithelial barrier function and visceral hyperalgesia in chronic stress remain unknown. **Methods:** Male rats were subjected to water avoidance stress for 10 consecutive days. Lubiprostone was administered daily during the stress phase. Visceromotor response to colorectal distension was measured. Human colon crypts and cell lines were treated with cortisol and lubiprostone. The transepithelial electrical resistance and FITC-dextran permeability were assayed. Chromatin immunoprecipitation was conducted to assess glucocorticoid receptor binding at tight junction gene promoters. **Key Results:** Lubiprostone significantly decreased chronic stress-induced visceral hyperalgesia in the rat ( $P < 0.05$ ;  $n = 6$ ). WA stress decreased occludin and claudin-1 and increased claudin-2 in rat colon crypts, which was prevented by lubiprostone. Cortisol treatment induced similar alterations of tight junction protein expression in Caco-2/BBE cells ( $P < 0.05$ ) and significantly changed paracellular permeability in monolayers ( $P < 0.01$ ). These changes were blocked by lubiprostone. Glucocorticoid receptor and its binding at occludin promoter region were decreased in cortisol-treated cells and human colon crypts, which was largely reversed by lubiprostone. In rat colonic cells, glucocorticoid receptor and its co-chaperone proteins were down-regulated after corticosterone treatment and lubiprostone reversed these changes. **Conclusions & Inferences:** Lubiprostone preferentially prevents chronic stress-induced alterations of intestinal epithelial tight junctions, barrier function, and visceral hyperalgesia that was associated with modulation of glucocorticoid receptor expression and function.

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## 1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder characterized by abdominal discomfort or pain and changes in bowel habit.<sup>1-3</sup> Increasing evidence suggests that disturbance of the intestinal epithelial barrier function contributes to diarrhea-prone IBS-related symptoms.<sup>4,5</sup> For example, the enhanced paracellular permeability is positively correlated with abdominal pain in IBS patients.<sup>6</sup> Recently, enhanced stress effects on gut barrier function have been implicated as a potential mechanism contributing to the pathophysiology of IBS.<sup>7,8</sup> Chronic stress has significant effects on gut physiology and pathophysiology including alterations in gastrointestinal motility, enhanced visceral pain perception (hyperalgesia), and impaired intestinal barrier function in animal models<sup>9,10</sup> and IBS patients.<sup>11,12</sup> The corticoid receptor is one of the major players in mediating many physiological events in response to acute and chronic stress.<sup>13,14</sup> The glucocorticoid receptor (GR), a ligand-activated transcription factor, depends on molecular chaperones for in vivo function.<sup>15</sup> After ligand binding, GR is translocated to the nucleus and binds to DNA on glucocorticoid response elements (GREs), recruiting transcriptional machinery and various coactivators and corepressors to positively or negatively regulate gene transcription. GR translocation is

mediated by the co-chaperone protein FK506 binding protein 5 (FKBP5) which is associated with the chaperone heat-shock protein 90 (Hsp90) to form a chaperon complex.<sup>16,17</sup> The Hsp90-GR complex, in its ATP-bound form, is stabilized by co-chaperone p23, whereas heat-shock protein 70 (Hsp70) mediates GR complex assembly and activity.<sup>15,18</sup> The importance of these chaperones has been established in the pathogenesis and therapy of stress and mental disorders.<sup>14,17,19</sup> In human colon epithelial cells, induction of Hsp70 expression displays a protective role in the epithelial barrier integrity which results in a decrease in paracellular permeability.<sup>20</sup> The augmentation of heat-shock proteins and reduced barrier integrity has been reported in colon tissues in an acute stress animal model.<sup>21</sup> The mechanism underlying how GR and its chaperones regulate intestinal paracellular permeability in chronic stress has not been determined. Lubiprostone, a bicyclic fatty acid derived from prostaglandin E1 (PGE1), activates chloride channel type 2 (CIC-2) in the apical membrane of epithelial cells<sup>22</sup> and promotes the passage of water into the luminal space and bowel movements which can improve symptoms associated with constipation.<sup>23,24</sup> CIC-2 channel plays an important role in the modulation of tight junctions by influencing caveolar trafficking of the tight junction protein occludin.<sup>25</sup> Targeted activation of CIC-2 chloride channels with lubiprostone stimulates repair of intestinal barrier function in the ischemia-injured porcine ileum and colon which is associated with impaired expression and function of tight junction proteins resulting in increased permeability.<sup>26</sup> However, it is unknown whether lubiprostone has a preventative effect on the increased permeability and enhanced visceral pain in animal models of chronic stress. The goal of the present study was to examine the hypothesis that lubiprostone prevents GR-mediated disruption of colon epithelial tight junction proteins and prevents increase of paracellular permeability and visceral pain.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals and water avoidance (WA) stress

Male Sprague-Dawley rats (weighing 160-180 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were housed in the animal facility that was maintained at 22°C with an automatic 12-hours light/dark cycle. The animals received a standard laboratory diet and tap water ad libitum.

All experiments were approved by the University of Michigan Committee on Use and Care of Animals according to National Institutes of Health guidelines. The animal study was conducted as shown in the following experimental scheme. Briefly, young-adult male rats were adapted in the animal facility for 3-5 days and then randomly grouped and subjected to 10-day WA stress as described previously.<sup>27</sup> The rats were placed on a glass platform in the middle of a tank filled with water (22°C) to 1 cm below the height of the platform. The animals were maintained on the tank for 1 hour in the morning (8 AM-10 AM) daily for 10 consecutive days. Lubiprostone (Takeda Pharmaceutical North America, Deerfield, IL, USA) was delivered orally instead of gavage to rats, to

**KEYWORDS** chronic stress; visceral hyperalgesia, glucocorticoid receptor, intestinal permeability, lubiprostone, tight junction

**Key Points**

- Chronic stress is associated with increased intestinal epithelial permeability and visceral hyperalgesia. Lubiprostone accelerates intestinal epithelial repair. It is unknown how lubiprostone regulates colon epithelial barrier function and visceral pain in chronic stress.
- Lubiprostone prevented chronic stress-induced differential alterations of epithelial tight junction proteins, down-regulation of glucocorticoid receptor and its chaperones, and increase in paracellular permeability and visceral pain in the rodent and human.
- Our findings support a novel mechanism for lubiprostone to regulate intestinal permeability and visceral pain.

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avoid distress or irritation, twice daily with 500  $\mu$ L medium-chain triglycerides (MCT) as described.<sup>28</sup> A dose of 10  $\mu$ g/kg body weight was suggested by the manufacturer and proved effective by a serial dose response pre-experiments. Rats were weighed each day for determination of the amount of lubiprostone delivered. All animals were treated with lubiprostone prior to stress procedure each day for a period of 10 days during the WA stress phase (WA+Lub group). MCT, the delivery medium for lubiprostone, was administered similarly to the SHAM control (MCT-CT) rats and WA stress (MCT-WA) rats. The MCT-CT rats were placed similarly for 1 hour daily for 10 days in a tank without water. The number of fecal output was recorded daily for each rat after 1 hour WA or SHAM stress. Behavioral assessments were conducted on the next day after completing the 10-day stress procedure. Separate groups of animals without behavioral measurements were used to harvest colon crypts from the distal colon, the same segment for balloon distention and visceral pain measurement. The aliquots of isolated colon crypts were used for biochemical and molecular characterization. The experimenter was blinded to animal treatment during behavioral experiments.

## 2.2 | Visceral motor response (VMR) to colorectal distention (CRD)

Visceral pain measurement was conducted as previously described.<sup>27,29</sup> Briefly, rats were deeply anesthetized with subcutaneous injection of a mixture of ketamine (60 mg/kg) and xylazine (5 mg/kg). An incision was made in the skin of the lower abdomen, and two perfluoroalkoxy-coated, 32-gauge stainless steel wires were inserted into the external oblique pelvic muscles superior to the inguinal ligament. Animals were injected with 500  $\mu$ L of 0.9% saline, and artificial tears eye ointment was placed onto their eyes. The animals were then allowed to recover for 3-5 days prior to the VMR measurement. Measurement of the VMR to CRD was conducted in awake animals on day 11, next day after the completion of 10-day WA stress procedure. The VMR was quantified by measuring activity of electromyography (EMG) in the external oblique musculature in the awake animals. CRD was conducted to constant pressures of 10, 20, 40, and 60 mm Hg by a custom-made distension control device. The responses were considered stable if there was <20% variability between 2 consecutive trials of CRD at 60 mm Hg. The increase in the area under curve (AUC), which is the sum of all recorded data points multiplied by the sample interval (in seconds) after baseline subtraction, was presented as the overall response during the course of the CRD test.

### 2.3 | Cell culture and treatment

The Caco-2/BBE colonic epithelial cell line, originally derived from a human adenocarcinoma, was obtained from Prof. David E Smith (University of Michigan, Ann Arbor, MI, USA). Cells were maintained at 50% density in DMEM containing 25 mmol/L glucose, 3.7 g/L NaHCO<sub>3</sub>, 4 mmol/L l-glutamine, 1% nonessential amino acids, 100 U/L penicillin/streptomycin (complete medium), supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St Louis, MO, USA) at 37°C in 10% CO<sub>2</sub>. For differentiation, cells were seeded on Transwell filters, 12 mm diameter, 0.4 µm pore diameter (Corning Inc, Lowell, MA, USA) at a density of 3.5 × 10<sup>5</sup> cells/cm<sup>2</sup> in complete medium for two days to allow the formation of a confluent cell monolayer. From day 3 after seeding, cells were kept in the same medium in both compartments (10% FBS Symmetric) and allowed to differentiate for 21 days with regular medium changes three times a week.<sup>30</sup> Rat colon epithelial FRC/TEX cell lines used in this study was a generous gift from Dr Kimberly Rieger-Christ, Sophia Gordon Cancer Center, Burlington, MA. These cells consist of two mycoplasma-negative rat colonic epithelial cell lines and the immortalized non-tumorigenic cell line.<sup>31</sup> The derivative transformed FRC/TEX cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), hydrocortisone (0.02 µg/mL), insulin (0.25 µg/mL), transferrin (0.12 µg/mL), glucose (67.5 µg/mL), and penicillin-streptomycin (50 U/mL) in a humidified environment at 37°C in the presence of 10% CO<sub>2</sub>.<sup>32</sup> For treatment, FRC/TEX and Caco-2/BBE Cells were incubated with GR agonist corticosterone (500 nmol/L) for 24 hours with/without lubiprostone (100 nmol/L; dissolved in DMSO). DMSO at the final 0.01% (v/v) concentration was used as the vehicle control. The cortisol dose at 500 nM was determined to mimic the serum level in the stressed rats after 10-day chronic WA stress as described previously.<sup>9</sup> Treated cells were then harvested for immunofluorescence staining and Western blot analysis.

2.4 | Human colon crypts culture and treatment Human colon crypts culture was conducted as described previously.<sup>33</sup> Briefly, colon biopsies were collected from healthy control patients in cold Dulbecco's minimal essential medium (Life Technologies, Carlsbad, CA, USA), supplemented with 2 mmol/L GlutaMax (Life Technologies), 50 µg/mL gentamicin (Life Technologies), 100 µg/mL

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normocin (InvivoGen, San Diego, CA, USA), and 2.5 µg/mL amphotericin (Life Technologies) at the University of Michigan Hospitals according to the procedures approved by the Institutional Review Board (IRB). The mucosa/submucosa was surgically separated from the muscularis propria and incubated in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) supplemented with the above antimicrobials for 15 minutes. The tissues were then incubated in 10 mmol/L dithiothreitol (DTT; Sigma-Aldrich) for 15 minutes, followed by treatment with 8 mmol/L ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) for 75 minutes.

Crypts were isolated and washed with cold keratinocyte growth medium gold (KGMG) and cultured in KGMG with Matrigel (BD Biosciences, San Jose, CA, USA) at a crypt density of 50-75 crypts/50  $\mu$ L Matrigel/2 cm<sup>2</sup> well. After cultured for 3 hours, crypts were treated with cortisol (500 nmol/L) with/without lubiprostone (100 nmol/L) for 24 hours. DMSO (0.01% v/v) was used as the vehicle control.

## 2.5 | Measurement of transepithelial electrical resistance (TEER) and FITC-Dextran permeability

The TEER assay was used to measure the effect of treatments on the integrity of the tight junctions between Caco-2/BBE cells as a model of human intestinal epithelium. Caco-2/BBE cells were seeded on 24well 12-mm polyester Transwell filters (Corning, Corning, NY, USA) with 0.4  $\mu$ m pore size at a concentration of  $2 \times 10^5$  cells/Transwell. Cells were grown in DMEM supplemented with 0.01 mg/mL human transferrin, 10% FBS, and pen/strep for 21 days until they formed a differentiated monolayer. For treatment, cortisol (500 nmol/L) was added to the apical site (upper chamber) of the transwell cultures since the expression of GR receptors is dominant at the apical epithelial cells in human colon crypts.<sup>34</sup> To determine the effect of cortisol, the TEER was measured before and after cortisol treatment for 24 hours in the presence and absence of lubiprostone (100 nm; inner/upper chamber) or DMSO (0.01% v/v) vehicle using an EVOM2 epithelial voltage meter (World Precision Instruments, Sarasota, FL, USA). After washing the cells, DMEM was dispensed into each filter in the apical and basolateral chamber; electrical probes were then immersed in the apical and basolateral chambers to measure the resistance of the monolayer. TEER was calculated after subtraction of the intrinsic resistance of the cell-free filter. To measure dextran permeability, fluorescein isothiocyanate (FITC)-dextran (4 kDa; 3 mg/mL) was added to the upper chamber without medium change. Aliquots were withdrawn from the lower chambers after 4 hours and assayed for fluorescence at 515 nm with excitation at 492 nm.

## 2.6 | Western blot analysis

Colon tissues in rats that had not undergone surgery or visceral pain measurement were dissected out next day after completing the 10-day WA stress procedure. The dissected colon segments were reversed inside-out and washed with 2 mmol/L DTT in cold PBS. Then, tissues were incubated with 4 mmol/L EDTA in cold PBS for 5 minutes with shaking. The epithelium layers were gently scrapped off with a forceps and collected in PBS, followed by centrifugation at 500 g for 5 minutes. The crypts aliquots containing epithelium were stored at  $-80^{\circ}\text{C}$  for later use or immediately homogenized in ice-cold lysis buffer containing 50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EGTA, 50 mmol/L NaF, 1.5 mmol/L MgCl<sub>2</sub>, 10% v/v glycerol, 1% v/v Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and Complete Protease Inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). In separate studies, rat FRC/TEX and human Caco-2/BBE cells were collected and homogenized using the same lysis buffer.

Proteins were separated and transblotted to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked and incubated with primary antibodies for GR (Cell Signaling, Danvers, MA, USA), mineralocorticoid receptor (MR; Santa Cruz Biotechnology, Santa Cruz, CA, USA), FKPB5 (Cell Signaling), Hsp90 (Cell Signaling); Hsp70 (Cayman Chemical, Ann Arbor, MI, USA), Hsp23 (p23; Cayman Chemical), claudin-1 & claudin-2 (Invitrogen Corp., Camarillo, CA, USA), occludin (Abcam, Cambridge, MA, USA), and  $\alpha$ -actin (Sigma-Aldrich) at 4°C, overnight, and subsequently with secondary antibodies (1:5000; Cell Signaling) for 1 hour at room temperature. The X-ray films were developed using SuperSignal West Dura Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Rockford, IL, USA).

## 2.7 | Quantitative PCR (qPCR)

Total RNA from rat colon crypt aliquots was isolated using the Trizol (Life Technologies, Grand Island, NY, USA) and RNeasy kit (Qiagen, Hilden, Germany). qPCR was performed using the BioRad iScript One-Step Reverse-Transcription PCR Kit with SYBR Green (Bio-Rad, Hercules, CA, USA) using the following specific primers from Integrated DNA Technologies (Coralville, IA, USA): claudin-1: forward-ATGACCCCTATCAATGCCAG, reverse-TGGTG TTGGGTAAGAGGTTG; claudin-2: forward-CAGCTCCGTTTTCTA GATGCC, reverse-TGCGGCTCTTGTTCCTTGGA; occludin: forward- AAAGCAGGGAAGGCCGAAG, reverse-TGTTGATCTGAAGTGATAGG TGG; GR: forward-GCGTCAAGTGATTGCAGCAGTGAA, reverse- GCAAAGCAGAGCAGGTTTCCACTT; GAPDH: forward-TGTGAACG GATTTGGCCGTA, reverse-TGAACTTGCCGTGGGTAGAG.

## 2.8 | Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed using a chromatin immunoprecipitation kit (EMD Millipore, Billerica, MA, USA). Briefly, the colon crypt aliquots or Caco-2/BBE cells were cross-linked using 1% formaldehyde and terminated by incubation with 0.125 mol/L glycine for 5 minutes. The cell lysate was incubated for 10 minutes at 4°C, and the crude nuclear extract was collected by centrifugation at 600 × g for 5 minutes at 4°C. The DNA was sonicated to random fragments between 200 bp and 500 bp. The chromatin was subjected to immunoprecipitation using the following antibodies: GR (#3660; Cell Signaling Technology, Danvers, MA, USA). Normal rabbit IgG was used as the control. DNA was eluted in elution buffer and used for PCR amplification.

### Primers

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for rat occludin promoter (forward-CATTTAATCAAACCTGGCAGC, reverse-CCTGGAGCTAGCAGAGTTAAAAGG) and for human occludin promoter (forward-CGACACACCACACCTACACT, reverse-ATGCGCACCAACGTGGAA) were obtained from Life Technologies (Grand Island, NY, USA). PCR was performed using Bio-Rad iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, Hercules, CA, USA).

## 2.9 | Immunofluorescence

FRC/TEX cells cultured on cover glasses were fixed for 30 minutes in 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Cells were then permeabilized with 0.3% Triton X-100 for 1 hour and then blocked with 10% normal goat serum in PBS with 0.3% Triton X-100 for 4 hours at room temperature. Primary antibodies used for overnight incubation were anti-occludin (1:1000) and anti-ZO1 (1:5000). Secondary antibodies Alexa Fluor 488 (1:500) and Alexa Fluor 594 (1:500) from Molecular Probes (Life Technologies) were used for incubation for 2 hour.

## 2.10 | Statistical analysis

To examine the VMR in response to CRD pressures, the EMG amplitudes, represented by calculating the AUC, were normalized as percentage of baseline response for the highest pressure (60 mm Hg) for each rat and then averaged for each group of rats. The effects of stress and/or lubiprostone treatment on the VMR to CRD were analyzed using a repeated-measures two-way ANOVA followed by Bonferroni posttest comparisons. Unpaired Student's t test was used to examine the data for protein, TEER, FITC-dextran, and ChIP assays. Results were expressed as means  $\pm$  SEM  $P < 0.05$  was considered statistically significant.

## 3 | RESULTS

### 3.1 | Lubiprostone prevented chronic stress-induced visceral hyperalgesia in the rat

In our previous studies, we demonstrated that chronic WA stress induced visceral hyperalgesia which was largely blocked by GR receptor antagonist RU-486.<sup>27,35</sup> It is unknown whether lubiprostone will have a preventative effect on chronic stress-associated visceral pain perception. In this study, we measured body weight gain, fecal pellet output and visceral pain in WA-stressed rats treated with lubiprostone or MCT vehicle. Intermittent 10-day WA stress induced a significant reduction in body weight gain in MCT-WA stress rats ( $29.0 \pm 2.2$  g) during this 10-day stress period compared to MCT-CT rats ( $43.5 \pm 2.8$  g) as shown in Figure 1A ( $P < 0.001$ ;  $n = 6-8$ ). Lubiprostone treatment prevented stress-induced reduction in body weight gain. The average number of fecal pellet output during 1-hour stress each day was  $0.78 \pm 0.19$  in MCT-CT rats, and it significantly increased to  $4.1 \pm 0.3$  in MCT-WA rats ( $P < 0.001$ ). Lubiprostone treatment did not affect the fecal pellet output compared to MCT-WA rats (Figure 1B). In MCT-WA rats, VMR in response to CRD, expressed as EMG activity, increased significantly to  $111.6\% \pm 13.8\%$  from the control level ( $64.9\% \pm 11.9\%$ ) of MCT-CT rats at 40 mm Hg pressure ( $P < 0.01$ ;  $n = 6-8$ ) and  $244.3\% \pm 23.5\%$  from the MCT-CT level ( $100.0\% \pm 9.2\%$ ) at 60 mm Hg pressure ( $P < 0.001$ ;  $n = 6-8$ ), respectively. In WA+Lub rats, VMR was significantly decreased to  $72.8\% \pm 7.3\%$  and  $155.6\% \pm 38.7\%$  at the distention pressures of 40 and 60 mm Hg, respectively, when compared to MCT-WA rat groups ( $P <$

0.05). No differences in VMR to 10 and 20 mm Hg distention pressures were observed in these three animal groups (Figure 1C). MCT vehicle did not significantly influence VMR to CRD at any distention pressure when EMG amplitudes were compared between MCT-CT rats and healthy control rats without any treatment (data not shown).

### 3.2 | Lubiprostone blocked chronic stress-induced alterations of intestinal epithelial tight junction proteins in the rat colon

To test whether lubiprostone treatment affects intestinal epithelial tight junctions in chronic stress, we performed quantitative PCR and immunoblot using the acutely harvested rat colon crypts. As shown in Figure 2A, chronic WA stress significantly decreased mRNA levels of claudin-1, occludin, and GR receptor in the colon crypts in MCT-WA rats compared to MCT-CT rats ( $P < 0.05$ ), while claudin-2 mRNA was significantly increased ( $P < 0.05$ ). Lubiprostone treatment during the stress phase significantly increased mRNA levels of occludin and claudin-2 in this WA+Lub rat group compared to MCT-WA rats ( $P < 0.01$ ), to the similar levels of MCT-CT rats, whereas it had modest effects on claudin-1 and GR receptor. Furthermore, Western blot showed that WA stress induced  $49.0\% \pm 11.9\%$  decrease in claudin-1 ( $P < 0.05$ ),  $73.8\% \pm 5.8\%$  decrease in occludin ( $P < 0.01$ ), and  $37.2\% \pm 6.3\%$  increase in claudin-2 protein ( $P < 0.05$ ) in the MCT-WA rat colon crypts compared to MCT-CT rats, respectively. Treatment with lubiprostone significantly prevented the decreases of occludin but not claudin-1 and increase of claudin-2 protein in the rat colon. The levels of occludin and claudin-2 in colon crypts in WA+Lub rats were reversed to  $84.3\% \pm 5.1\%$  ( $P < 0.05$ ) and  $61.9\% \pm 6.5\%$  ( $P < 0.01$ ) of their corresponding levels (100%) of MCT-CT rats, respectively, after lubiprostone treatment as shown in Figure 2B,C. Consistent with the quantitative PCR result for claudin-1, lubiprostone had a modest reversal effect on chronic stress-induced down-regulation of claudin-1.

**FIGURE 1** Effect of lubiprostone (Lub) on chronic WA stress-induced visceral hyperalgesia in the rat. A, Body weight gain after 10-day WA or SHAM stress in rats treated with lubiprostone or MCT vehicle. B, Averaged number of fecal pellet output during 1 hour WA or SHAM stress period in MCT-CT, MCT-WA, and WA+Lub rat group. C, EMG amplitude, expressed as AUC of the VMR to CRD, was significantly increased following 10-day WA stress compared with the controls. Lubiprostone treatment largely prevented WA stress-induced increase in VMR to CRD at 40 and 60 mm Hg pressures. Data are expressed as mean  $\pm$  SEM,  $n = 6-8$  in each group. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  for MCT-WA rats compared to the MCT-CT rats. # $P < 0.05$  for lubiprostone-treated stressed rats (WA+Lub) compared to MCT-WA rats without lubiprostone treatment.

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### 3.3 | Effects of lubiprostone on cortisol-induced changes in tight junctions in human colon crypts and Caco-2/BBE cells

To test whether disruption of tight junctions observed in chronically stressed rats can be reproduced in human cells, differentiated Caco-2/BBE cells and human colon crypts derived from healthy controls were treated with cortisol. The optimal dose for cortisol treatment was determined by treating cells with serial concentrations of cortisol for 24 hours. As shown in Figure 3A, occludin protein decreased in a dose-dependent manner in these cells after cortisol treatment and the dose of 500 nmol/L cortisol was used for the subsequent experiments. As shown in Figure 3B,C, cortisol treatment induced  $53.8\% \pm 7.5\%$  decrease in claudin-1 ( $P < 0.05$ ),  $45.5\% \pm 9.4\%$  decrease in occludin ( $P < 0.05$ ), and  $171.3\% \pm 24.3\%$  increase in claudin-2 ( $P < 0.01$ ) compared with the control groups, respectively, in differentiated Caco-2/BBE cells after cortisol treatment. Lubiprostone significantly prevented these changes caused by cortisol. The protein levels of claudin-1, occludin, and claudin-2 were  $125.5\% \pm 9.3\%$ ,  $92.0\% \pm 5.3\%$ , and  $191.3\% \pm 32.9\%$ , respectively, in cortisol-treated cells in the presence of lubiprostone compared to their corresponding control levels. The differences between cortisol-treated cells with and without lubiprostone were significant ( $P < 0.05$ ). As shown in Figure 3D,E, human colon crypts demonstrated  $55.7\% \pm 4.4\%$  decrease in claudin-1 ( $P < 0.01$ ),  $33.7\% \pm 9.9\%$  decrease in occludin ( $P < 0.05$ ), and  $70.7\% \pm 13.5\%$  increase in claudin-2 ( $P < 0.01$ ), respectively, after cortisol treatment compared with the control groups. Co-treatment with lubiprostone significantly prevented the decreases of claudin-1 and occludin and the increase of claudin-2. The levels of claudin-1, occludin, and claudin-2 in lubiprostone and cortisol treated were  $73.8\% \pm 13.1\%$ ,  $81.4\% \pm 7.04\%$ , and  $147.1\% \pm 8.6\%$  of their corresponding control levels, respectively ( $P < 0.05$ ).

#### 3.4 | Effects of lubiprostone on paracellular permeability in Caco-2/BBE cells

In differentiated Caco-2/BBE cell monolayers, TEER was measured before and after treatment with cortisol in the presence and absence of lubiprostone, while FITC-dextran leakiness was measured after cortisol treatment with/without lubiprostone. As shown in Figure 4A, change of TEER decreased  $24.9 \pm 5.9$  ohms/cm<sup>2</sup> in Caco-2/BBE monolayers after cortisol treatment for 24 hours, whereas it increased  $14.8 \pm 7.1$  ohms/cm<sup>2</sup> in the vehicle controls. The difference between these two groups was significant ( $P < 0.01$ ;  $n = 18$ ). In the presence of lubiprostone, change of TEER increased  $28.4 \pm 9.4$  ohms/cm<sup>2</sup> in cortisol-treated cells, which was significantly different compared to those without lubiprostone co-treatment ( $P < 0.001$ ;  $n = 18$ ). Moreover, FITC-dextran measurement in the differentiated Caco-2/BBE monolayers showed  $40.7\% \pm 2.5\%$  increase in the intensity of 4 kDa FITC-dextran in the lower chamber of the Transwell cell cultures compared to the controls ( $P < 0.01$ ;  $n = 12$ ). This enhancement was reduced significantly to  $122.7\% \pm 4.8\%$  of the control by treatment with lubiprostone ( $P < 0.01$ ; Figure 4B).

#### 3.5 | Lubiprostone prevented cortisol-induced down-regulation of GR in human samples

We reported previously that GR was down-regulated after cortisol treatment in Caco-2/BBE cells.<sup>34</sup> Here, we examined

**FIGURE 2** Lubiprostone treatment differentially altered epithelial tight junction protein expression in the rat colon in chronic stress. A, Quantitative PCR analysis of the expression levels of tight junction genes including claudin-1, claudin-2, and occludin and the expression of GR receptor in colon crypts of MCT-WA rats compared to MCTCT rats in the presence and absence of lubiprostone. B, Immunoblot for detection of claudin-1, claudin-2, and occludin proteins in the colon crypts in MCT-CT, MCT-WA, and WA+Lub rats. C, Bar graph depicting the relative protein levels of claudin-1, claudin-2, and occludin in WA+Lub rats compared to MCT-WA rats and MCT-CT rats. n = 4-6 of each group. \*P < 0.05; \*\*P < 0.01 for MCT-WA rats compared to MCT-CT rats. #P < 0.05; ##P < 0.01 for WA+Lub rats compared to MCT-WA rats without lubiprostone treatment.

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whether lubiprostone treatment altered GR expression. As shown in Figure 5A, the protein level of GR in differentiated Caco-2/BBE cells was significantly decreased to  $36.6\% \pm 4.7\%$  after cortisol treatment for 24 hours ( $P < 0.01$ ). Administration of lubiprostone during cortisol treatment reversed GR expression to  $52.6\% \pm 7.5\%$  of the untreated level ( $P < 0.05$ ). Similarly, GR protein expression decreased  $64.1\% \pm 5.3\%$  in acutely harvested human colon crypts after cortisol treatment for 24 hours ( $P < 0.01$ ) as shown in Figure 5B. Lubiprostone significantly reversed GR protein level to  $113.5\% \pm 10.9\%$  of the untreated control ( $P < 0.01$ ). In addition, we examined GR protein levels in colon crypts from rats subjected to 10-day WA stress with/without lubiprostone. As shown in Figure 5C, GR protein was significantly decreased in colon crypts in MCT-WA rats compared to MCT-CT rats ( $P < 0.001$ ; n = 5). Lubiprostone treatment partially reserved GR protein level (to  $70.6\% \pm 21.5\%$ ) in WA+Lub group compared to the level of  $40.1\% \pm 14.4\%$  in MCT-WA group. The difference between these two groups was not significant ( $P = 0.137$ ; n = 5).

**3.6 | Lubiprostone reversed cortisol-induced downregulation of GR co-chaperones**  
We examined GR chaperon proteins in differentiated Caco-2/BBE cells to test the hypothesis that GR function can be regulated by lubiprostone. As shown in Figure 6, both cortisol and lubiprostone did not alter the levels of GR chaperon proteins Hsp90 and Hsp70. In contrast, GR co-chaperon proteins p23 and FKBP5 were significantly decreased to  $40.7\% \pm 6.6\%$  ( $P < 0.01$ ) and  $79.8\% \pm 10.5\%$  ( $P < 0.05$ ) of the control levels, respectively, in Caco-2 cells treated by cortisol. Lubiprostone co-treatment significantly blocked cortisol-induced down-regulation of p23 and FKBP5, suggesting the regulatory effect of lubiprostone on GR expression and function.

**3.7 | Lubiprostone inhibited cortisol-induced decrease in GR binding to occludin gene promoter**

Next, we tested whether the binding of GR, as a positive transcription factor, to occludin promoter regions was affected by cortisol and

**FIGURE 3** Differential effects of cortisol and lubiprostone on tight junction proteins in differentiated Caco-2/BBE cells and acutely harvested human colon crypts. A, Dose response effect of cortisol treatment (0-1000 nmol/L; 24 hours) on occludin expression in Caco-2/BBE cells. B, Differential changes of claudin-1, claudin-2, and occludin in Caco-2/BBE cells treated by 500 nmol/L cortisol for 24 hours in the presence and absence of lubiprostone (100 nmol/L). C, Statistical analysis of tight junction protein levels in Caco-2/BBE cells after cortisol and lubiprostone treatment. D, Immunoblots for tight junction proteins in acutely harvested colon crypts from healthy control patients after treatment with cortisol (500 nmol/L) and/or lubiprostone (100 nmol/L) for 24 hours. E, Statistical analysis showed differential effects of cortisol on claudin-1, claudin-2 and occludin protein expression in human colon crypts with/without lubiprostone treatment. n = 3-4. \*P < 0.05; \*\*P < 0.01 for cortisol treatment compared to the controls. #P < 0.05; ##P < 0.01 for cortisol and lubiprostone-treated cells compared to the cortisol-treated cells.

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lubiprostone. As shown in Figure 7A, ChIP analysis revealed that the binding of GR protein to occludin gene promoter region was decreased in colon crypts in MCT-WA rats compared to MCT-CT rats.

Treatment with lubiprostone during the stress phase prevented the decrease of GR binding to the occludin promoter, which is consistent with the effect of lubiprostone on chronic stress-induced down-regulation of occludin protein expression in the rat colon. In differentiated Caco-2/BBE cells, the relative amount of GR binding to occludin promoter decreased substantially treated by cortisol compared with controls. Lubiprostone treatment prevented the decrease of GR binding to the occludin promoter caused by cortisol administration as shown in Figure 7B.

**3.8 | Lubiprostone prevented corticosterone-induced alterations of GR chaperon protein in the rat colonic cells**

In rat colonic FRC/TEX cells, occludin expression and localization were significantly disrupted after corticosterone treatment and this change was prevented by lubiprostone treatment (Figure 8A).

In contrast, ZO-1 expression and localization were not altered by cortisol treatment. As shown in Figure 8B, GR protein level was decreased in corticosterone-treated FRC/TEX cells which was largely reversed by lubiprostone, whereas MR was not affected by corticosterone. Corticosterone had no significant effect on GR chaperon protein Hsp90, but decreased protein levels of GR co-chaperon proteins p23 and FKBP5. Lubiprostone treatment normalized the down-regulation of p23 and FKBP5 in these cells.

#### 4 | DISCUSSION

In this study, we revealed a novel role for lubiprostone to ameliorate stress-induced disruption of intestinal epithelial tight junction protein expression and function in the rodent and human colon that was associated with regulation of GR receptor expression and function. Our data demonstrate that lubiprostone prevents chronic WA stress-induced down-regulation of occludin and up-regulation of claudin-2 in rat colon crypts and significantly reduced visceral hyperalgesia observed in the stressed rats. Stress-induced alterations of claudin-1, claudin-2, and occludin were reproduced in

Caco-2/BBE cells and control human colon crypts treated with cortisol, an agonist for GR receptor. Lubiprostone blocked these changes and prevented cortisol-induced epithelial barrier dysfunction in Caco-2/BBE monolayers. Furthermore, GR expression was decreased in cortisol-treated Caco-2/BBE cells and human colon crypts, which was largely reversed by lubiprostone. ChIP-qPCR assay revealed that lubiprostone normalized decreased GR binding at occludin promoter region under stress conditions. In addition, GR and its co-chaperone proteins p23 and FKBP5 were down-regulated after corticosterone treatment in rat colonic cells and lubiprostone prevented these changes. Intestinal epithelial tight junction protein claudins have two different functional subcategories affecting paracellular permeability. One type of claudins, such as claudin-1, acts as “sealing” molecules which tighten the paracellular pathway against ions and larger solutes and maintain the integrity of the paracellular tight junctions with other tight junction proteins, that is, occludin. Disruption of these claudins and occludin expression decreases transepithelial electric resistance (TEER) and increases paracellular permeability. The other type of claudins, such as claudin-2, function as “pore-forming” molecules which enhance paracellular permeability in a charge-selective fashion.<sup>36,37</sup> For example, claudin-1 contributes to maintaining a high TEER in cell culture models and is indispensable for creating and maintaining the epidermal barrier.<sup>38,39</sup> In contrast, over-expression of claudin-2 induces lower transepithelial resistance in kidney cells and increases paracellular permeability.<sup>40,41</sup> In our study, we observed that chronic WA stress induced the reduction in the protein levels of claudin-1 and occludin and increase in the protein level of claudin-2 in colonic epithelium in rats. This is consistent with our previous report that corticosterone induced down-regulation of tight junction proteins and increase in colon epithelial permeability

**FIGURE 4** Paracellular permeability measurements in differentiated Caco-2/BBE monolayers in the presence and absence of cortisol and lubiprostone. **A**, The changes of transepithelial electrical resistance (TEER) before and after treatment with cortisol (500 nmol/L) and/or lubiprostone (100 nmol/L) for 24 hours in Caco-2/BBE cells cultured in Transwells for 21 days. n = 18. **(B)** FITC-dextran (4 kDa) permeability measurement in 21-day cultured Caco-2/BBE cells after 500 nmol/L cortisol treatment for 24 hours in the presence and absence of lubiprostone (100 nmol/L). n = 12. \*\*P < 0.01 for cortisol-treated cells compared to the controls. #P < 0.05; ###P < 0.001 for cortisol and lubiprostone-treated cells compared to the cortisol-treated cells.

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in the rats<sup>9</sup> and that chronic stress resulted in impairment of intestinal barrier function and visceral hyperalgesia.<sup>9,10</sup> It has been reported that claudin-2, which is sensitive to stressful conditions, is involved in paracellular water transport and contributes 23%-30% of the total water transport in proximal kidney tubules.<sup>42,43</sup> The expression of pore-forming claudin-2 could be induced by tumor necrosis factor-neuropeptide Y “cross-talk” in vitro which was associated with increased epithelial permeability in inflammation.<sup>44</sup> In normal colon, claudin-2 is highly expressed in the crypt bottom containing the undifferentiated and proliferative colonocytes.<sup>45</sup> However, the detailed function of claudin-2 in the colon is largely unknown, particularly under chronic stress conditions. We

hypothesize that chronic stress-induced up-regulation of claudin-2 in the colon can lead to increased water secretion and epithelial permeability and that prevention of claudin-2 increase by lubiprostone can normalize chronic stress-induced disruption of intestinal barrier function. It has been

**FIGURE 5** Effects of lubiprostone on GR receptor protein expression in differentiated Caco-2/BBE cells, human colon crypts, and stressed rats. A, Protein expression of GR receptor in 21-day cultured Caco-2/BBE cells treated by cortisol (500 nmol/L) and lubiprostone (100 nmol/L) for 24 hours. B, Cortisol treatment (500 nmol/L; 24 hours) significantly decreased GR protein level in human colon crypts. Lubiprostone (100 nmol/L) prevented cortisol-induced down-regulation of GR receptor. C, GR protein expression in colon crypts isolated from MCT-CT, MCT-WA, and WA+Lub rat groups. n = 3-5. \*\*P < 0.01 for cortisol-treated group compared to the controls. \*\*\*P < 0.01 for MCT-WA rats compared to MCT-CT rats. #P < 0.05; ##P < 0.01 for cortisol and lubiprostone-treated group compared to the cortisol-treated group.

**FIGURE 6** Differential effects of cortisol and lubiprostone on the alterations of GR chaperone proteins in differentiated Caco-2/BBE cells. A, Immunoblots for probing the express levels of GR chaperone and co-chaperone proteins including Hsp90, Hsp70, p23, and FKBP5 in 21-day cultured Caco-2/BBE cells in the presence and absence of cortisol (500 nmol/L; 24 hours) and lubiprostone (100 nmol/L; 24 hours). B, Statistical bar graph showed that cortisol treatment decreased p23 and FKBP5 in Caco-2/BBE cells and lubiprostone treatment largely blocked down-regulation of p23 and FKBP5 induced by cortisol. n = 3. \*P < 0.05; \*\*P < 0.01 for cortisol-treated cells compared to the controls. #P < 0.05; ##P < 0.01 for cortisol and lubiprostone-treated group compared to the cortisol-treated group.

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reported that claudin-2 is increased in the jejunum in IBS with diarrhea,<sup>46</sup> supporting an important role of this tight junction protein in regulation of intestinal barrier function. In our previous study, we observed significant decrease in GR receptor expression in colon epithelial cells in the stressed and corticosterone-treated rats, which was largely blocked by corticoid receptor antagonist RU-486.<sup>9</sup> In this study, we further demonstrated that GR protein was down-regulated in differentiated Caco-2/BBE cells and normal human colon crypts after cortisol treatment that was associated with increased paracellular permeability, suggesting a role for GR in modulation of tight junction protein expression and function. These results are consistent with other studies using in vitro cell lines.<sup>47,48</sup> Our ChIP-qPCR analysis showed that GR could directly bind to occludin promoter and this GR binding was decreased in the colon crypts in WA-stressed rats, indicating a direct regulatory role of GR, as a positive transcription factor, for occludin transcription and expression. Restoration of GR protein by lubiprostone thus reserved the decrease in GR transcriptional binding to occludin promoter induced by chronic stress, increased occludin transcription and expression, and subsequently normalized paracellular permeability. Similar regulatory role of GR for claudin-1 was reported in our recent study.<sup>34</sup> Interestingly, chronic stress-induced decrease in GR expression was prevented by lubiprostone in Caco-2/BBE cells and human colon crypts, but not in chronic WA stress rat model which showed a modest effect. One possible explanation is the complexity of in vivo rat stress model

compared to the relatively simple in vitro model using cultured cells/crypts.

Adrenocorticotrophic hormone, at systemic level, and several neuropeptides, at local level, can be released in the animal model under chronic stress conditions, which may modulate GR transcriptional binding to occludin and claudin-1 promoter regions in the presence of lubiprostone. Secondly, we observed large variability in down-regulation of GR protein in stressed rats and in stressed rats treated with lubiprostone. The difference in baseline levels of GR receptor, tight junction proteins, and their epigenetic regulatory status in healthy control rats, including DNA methylation in gene promoter regions, may contribute to the differential effects observed in animals subjected to chronic stress and lubiprostone treatment. Further studies are needed to dissect the detailed regulatory pathways in stress axis that are involved in regulation of differential restoration of disrupted epithelial tight junction proteins by lubiprostone. Like many signaling proteins, the function of GR depends on the coordination of Hsp90 and Hsp70 chaperone cycles. Full recovery of ligand binding to GR requires ATP hydrolysis on Hsp90 and the Hop and p23 co-chaperones.<sup>15</sup> In our study, both Hsp90 and Hsp70 were not affected by GR agonist, cortisol/corticosterone, in Caco-2/BBE and FRC/TEX cells. However, p23 expression was significantly down-regulated in the colon epithelial cells in stressed rats and colonic cell lines treated with glucocorticoids, suggesting the recovery of GR ligand binding was significantly incapacitated under chronic stress conditions. In addition, FKBP5, a Hsp90 cochaperone that regulates GR function,<sup>49</sup> was decreased in glucocorticoid-treated colonic cells. This result is consistent with the

**FIGURE 7** Chromatin immunoprecipitation (ChIP) followed by PCR analysis for transcription factor GR binding to occludin promoter regions in rat colon crypts and differentiated Caco-2/BBE cells. A, ChIP using anti-GR antibody followed by PCR for occludin promoter in colon crypts isolated from MCT-CT, MCT-WA, WA+Lub rats. PCR neg: no input DNA; ChIP neg: ChIP with normal rabbit IgG. Input: 4% input DNA from relevant rat crypt samples for normalization. B, ChIP and PCR analysis of GR binding to occludin promoter region in differentiated Caco-2/BBE cells treated by cortisol (500 nmol/L) for 24 hours in the presence and absence of lubiprostone (100 nmol/L)

**FIGURE 8** Effects of corticosterone (CORT) and lubiprostone on occludin, GR, and GR chaperone proteins in rat colonic FRC/TEX cells. A, Immunofluorescence for occludin and ZO-1 expression in FRC/TEX cells treated by corticosterone (500 nmol/L) for 24 hours in the presence and absence of lubiprostone (100 nmol/L). The expression and localization of occludin but not ZO-1 was significantly disrupted in corticosterone-treated cells compared to the controls. Lubiprostone largely reversed this change. B, Immunoblots for GR, MR, and GR chaperone proteins Hsp90, p23, and FKBP5 in 500 nmol/L corticosterone-treated FRC/TEX cells for 24 hours with/without lubiprostone (100 nmol/L). n = 3

previous report that FKBP5 links to the development of posttraumatic stress disorder (PTSD) and was found to be less expressed in PTSD.<sup>50</sup> These data strongly support a function of FKBP5 as a modulator of the response to stressful life events. It is very likely that FKBP5 functions in chronic stress through its action in GR resistance and translocation.<sup>17</sup> Accordingly, down-regulation of GR altered the transcription and expression of epithelial tight junction proteins and disrupted barrier function under chronic stress conditions and subsequently induced visceral hyperalgesia in stressed animals. However, the exact mechanism underlying how these co-chaperones modulate GR ligand binding and translocation to the nucleus in colon epithelial cells in chronic stress remains to be elucidated. It has been reported that lubiprostone significantly improves symptoms in patients with chronic constipation<sup>51</sup> and is more effective than placebo in improving abdominal pain or bloating in IBS patients with constipation.<sup>52</sup> The signal transduction pathway includes activation of CIC-2 channels resulting in a chloride-rich fluid secretion, activation of prostaglandin E type receptors with increase in mucus/fluid secretion, and restitution of epithelial cell barrier properties.<sup>26,28,51</sup> The current study demonstrates that lubiprostone significantly reduced chronic stress-induced visceral hyperalgesia in the rat, which is consistent with the effects reported in IBS patients.<sup>52</sup> This beneficial effect of lubiprostone most likely contributes to the prevention of chronic stress-induced changes in epithelial tight junction proteins including claudin-1, claudin-2, and occludin, and subsequent recovery of paracellular permeability in both in vivo and in vitro models. Importantly, our study further demonstrated a novel role for lubiprostone to regulate epithelial barrier function through modulation of GR expression and function. Lubiprostone significantly prevented down-regulation of GR co-chaperones including p23 and FKBP5 induced by chronic stress, suggesting a signaling modulation through GR ligand binding recovery and nucleus translocation. The prevention of down-regulation in GR results in reconstitution of GR binding to tight junction gene promoter regions and increases epithelial cell tight junction gene transcription, protein expression, and thereby restores epithelial cell barrier integrity and function. On the other hand, heat-shock proteins have been reported to regulate chloride channel expression and function.<sup>53</sup> Recovery of p23 and FKBP5 by lubiprostone may, therefore, lead to enhancement of chloride channel activity and fluid secretion to reduce chronic stress-induced symptoms. In our study, we cannot exclude the possibility that lubiprostone may also directly activate CIC-2 chloride channels<sup>25,54</sup> since lubiprostone was gavage-delivered in rat models and administered in the inner chamber of transwell cultures of Caco-2 cells. However, the fact that lubiprostone does not affect fecal pellet output in stressed rats does not support the involvement of direct activation of CIC-2 channels in this animal model. It is noteworthy that GR co-chaperone p23 is also identified as prostaglandin E synthase 3, a cytosolic prostaglandin E2 synthase. The recovery of the expression and function of this prostaglandin E synthase may increase the production of prostaglandin E2, activate prostaglandin EP receptors, and downstream signaling pathways that promote intestinal epithelial restoration.<sup>55</sup> In summary, our results support the novel concept that lubiprostone has a protective role in chronic stress-induced impairment in intestinal epithelial cell tight junction protein expression and function resulting in visceral hyperalgesia. These actions of lubiprostone appear to be attributable to the prevention of stress-induced disruption of intestinal epithelial tight junction protein expression and maintenance of intestinal barrier function associated with modulation of

GR receptor expression and function. GR, acting as a positive transcription factor for claudin-1 and occludin, regulates tight junction gene transcription and expression. Chronic stress-induced downregulation of GR receptor, therefore, leads to GR-mediated decreases in tight junction proteins claudin-1 and occludin and increase in claudin-2, resulting in increased paracellular permeability in animal models and cell lines. Lubiprostone treatment significantly prevents stress-induced down-regulation of GR receptor and its chaperone proteins, which promotes the recovery of GR-mediated regulation of tight junction protein expression, paracellular permeability, and visceral pain perception. These observations may help guide future drug development in this area by providing a rationale to target pathways that either prevent impairment and/or promote restoration in intestinal epithelial cell tight junction protein expression and function.

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#### 20. Which usually will NOT cause gut dysbiosis?

- a. Reduction of beneficial microbes
- b. Probiotics
- c. Overgrowth of pathogenic species
- d. Decreased diversity of microbes

#### The Causes of Intestinal Dysbiosis: A Review

Jason A. Hawrelak, BNat (Hons), PhD Candidate and Stephen P. Myers, PhD, BMed, ND role in both excluding these macromolecules and microbes from the systemic circulation and absorbing crucial nutrients.<sup>2</sup> As mentioned above, the mucosa is exposed to bacterial products – endotoxins,<sup>3</sup> hydrogen sulphide,<sup>4</sup> phenols, ammonia, and indoles<sup>5</sup> – that can have detrimental effects on both mucosal and host health.<sup>5</sup> The presence of many of these toxic metabolites is directly dependent on the type of fermentation that occurs in the bowel. In turn, this fermentation is dependent on the type of bacteria present in the bowel, as well as the substrates available for fermentation. Diets high in protein<sup>6</sup> and sulfate (derived primarily from food additives)<sup>4</sup> have been shown to contribute greatly to the production of these potentially toxic products. The production and absorption of toxic metabolites is referred to as bowel toxemia.<sup>7</sup> The bowel toxemia theory has historical roots extending as far back as Hippocrates. In 400 B.C. he stated that, “...death sits in the bowels...” and “...bad digestion is the root of all evil...”<sup>8</sup> More modern proponents of the bowel toxemia theory have included naturopath Louis Kuhne in the late nineteenth century,<sup>9</sup> as well as naturopath Henry Lindlahr<sup>10</sup> and Nobel prize laureate Elie

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### Intestinal Dysbiosis

Metchnikoff in the early twentieth century.<sup>11</sup> Louis Kuhne proposed that excess food intake, or the intake of the wrong types of food, resulted in the production of intestinal toxins. Fermentation of these toxins resulted in increased growth of bacteria within the bowel and, subsequently, disease. He believed a predominantly vegetarian and mostly raw diet would prevent build-up of intestinal toxins and, hence, would prevent and even cure disease.<sup>9</sup> Only a few years later, Metchnikoff popularized the idea that fermented milk products could beneficially alter the microflora of the GIT. He believed many diseases, and even aging itself, were caused by putrefaction of protein in the bowel by intestinal bacteria. Lactic acid-producing bacteria were thought to inhibit the growth of putrefactive bacteria in the intestines. Thus, yogurt consumption was recommended to correct this “auto-intoxication” and improve composition of the microflora.<sup>11,12</sup> The bowel toxemia theories eventually evolved into the intestinal dysbiosis hypothesis. The term “dysbiosis” was originally coined by Metchnikoff to describe altered pathogenic bacteria in the gut.<sup>13</sup> Dysbiosis has been defined by others as “...qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution.”<sup>14</sup> Thus dysbiosis is a state in which the microbiota produces harmful effects via: (1) qualitative and quantitative changes in the intestinal flora itself; (2) changes in their metabolic activities; and (3) changes in their local distribution. The dysbiosis hypothesis states that the modern diet and lifestyle, as well as the use of antibiotics, have led to the disruption of the normal intestinal microflora. These factors result in alterations in bacterial metabolism, as well as the overgrowth of potentially pathogenic microorganisms. It is believed the growth of these bacteria in the intestines results in the release of potentially toxic products that play a role in many chronic and degenerative diseases.<sup>13</sup> There is a growing body of evidence that substantiates and clarifies the dysbiosis theory. Altered bowel flora is now believed to play a role in myriad disease conditions, including GIT disorders like irritable bowel syndrome (IBS)<sup>15</sup> and inflammatory bowel disease (IBD),<sup>16,17</sup> as well as more systemic conditions such as rheumatoid arthritis (RA)<sup>18</sup> and ankylosing spondylitis.<sup>19</sup> Thus, knowledge of the factors that can cause detrimental changes to the microflora is becoming increasingly important to the clinician.

**The Importance of Normal GIT Microflora** The microflora of the gastrointestinal tract represents an ecosystem of the highest complexity.<sup>14</sup> The microflora is believed to be composed of over 50 genera of bacteria<sup>20</sup> accounting for over 500 different species.<sup>21</sup> The adult human GIT is estimated to contain 10<sup>14</sup> viable microorganisms, which is 10 times the number of eukaryotic cells found within the human body.<sup>22</sup> Some researchers have called this microbial population the “microbe” organ – an organ similar in size to the liver (1-1.5 kg in weight).<sup>23</sup> Indeed, this microbe organ is now recognized as rivaling the liver in the number of biochemical transformations and reactions in which it participates.<sup>24</sup> The microflora plays many critical roles in the body; thus, there are many areas of host health that can be compromised when the microflora is drastically altered. The GIT microflora is involved in stimulation of the immune system, synthesis of vitamins (B group and K), enhancement of GIT motility and function, digestion and nutrient absorption, inhibition of

pathogens (colonization resistance), metabolism of plant compounds/drugs, and production of short-chain fatty acids (SCFAs) and polyamines.<sup>14,25,26</sup>

Factors that Can Alter the GIT Microflora Many factors can harm the beneficial members of the GIT flora, including antibiotic use, psychological and physical stress, radiation, altered GIT peristalsis, and dietary changes. This review will focus exclusively on the interactions of antibiotics, stress, and diet with the gut flora.

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Table 1a. The Effects of Some Selected Antibiotics on GIT Microflora

Agent

Ampicillin

Ampicillin/ Sulbactam

Amoxicillin

Amoxicillin/ clavulanic acid

Azlocillin

Aztreonam

Bacampillicin

Cefaclor

Cefaloridine

Cefazolin

Cefbuperazone

Cefixime

Cefmenoxime

Enterobacteria

↓↓

↓

↓

-

↓

↓↓

-

-

-

-

↓↓

↓↓

↓

Enterococci

↓↓

↓

↓

-

↓

↑

-

-

-

-

↓

↓

-

Anaerobic bacteria

↓↓

↓

↓

-

↓

-

↓

-

-

-

↓↓

↓↓

-

Overgrowth of resistant strains

+

+

+

+

+

+

-

+

-

+

-

+

+

Days to normalization of flora (postadministration)

not stated

14

not stated

not stated<sup>29</sup>

not stated

14<sup>29</sup>

not stated

7

not stated<sup>29</sup>

not stated<sup>29</sup>

28

14

not stated

Other

∇ in Lactobacilli and Bifidus; ↑ in Candida; ∇ production of SCFAs<sup>29,36,48</sup>

∇ in Lactobacilli and Bifidus<sup>29,48</sup>

↑ in Candida<sup>29</sup>

∇ in Lactobacilli<sup>29</sup>

No significant change in Lactobacilli, Bifidus or yeasts<sup>29,48</sup>

∇ in Bifidus; ↑ in *C. difficile*<sup>29</sup>

∇ in Lactobacilli and Bifidus<sup>29</sup>

∇ in Bifidus; ↑ in *C. difficile*<sup>29</sup>

∇ in Lactobacilli and Bifidus; ↑ in Candida and Clostridia<sup>29</sup>

Impact on

An overview of some of the research investigating the effects of selected antibiotics on the GIT microflora. ↓↓ = strong suppression (> 4 log 10 CFU/g feces); ↓ = mild to moderate suppression (2-4 log 10 CFU/g feces); ↑ = increase in number of organisms during therapy; - = no significant change; ∇ = decrease; + = positive result; Bifidus = Bifidobacterium spp.

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Table 1b. The Effects of Some Selected Antibiotics on GIT Microflora

Agent

Cefoperazone

Cefotaxime

Cefotetan

Cefotiam

Cefoxitin

Ceftazadime

Ceftizoxime

Ceftriaxone

Cephradine

Cephrocile

Enterobacteria

↓↓

↓

↓

↓

↓

↓

↓

↓↓

-

↑  
Enterococci

↓↓

↓

↑

-

↑

-

-

↓↓

-

↓

Anaerobic bacteria

↓

-

↓

-

↓

-

-

↓

-

-

Overgrowth of resistant strains

+

-

+

+

+

-

+

+

-

+

Days to normalization of flora (postadministration)

not stated

not stated<sup>29</sup>

not stated

not stated

not stated

not stated

not stated

28

not stated

4

Other

∇ in Lactobacilli and Bifidus; ↑ in *C. difficile* and *Candida*; 70% of drug excreted in bile<sup>29,48,49</sup>

∇ in Lactobacilli; ↑ in *C. difficile*<sup>29</sup>

↑ in *Candida* and *Pseudomonas*; ∇ in Lactobacilli<sup>29</sup>

∇ in Lactobacilli and Bifidus; ↑ in *C. difficile* and *Candida*<sup>29,48</sup>

∇ in Lactobacilli<sup>29</sup>

No effect on Lactobacilli; ↑ in *Citrobacter* spp. and *Proteus* spp.<sup>29</sup>

∇ in Bifidus; ↑ in *Candida*; 30% of drug excreted in bile<sup>29,49</sup>

No ↑ in yeast<sup>29</sup>

↑ in *C. difficile*<sup>29</sup>

Impact on

An overview of some of the research investigating the effects of selected antibiotics on the GIT microflora. ↓↓ = strong suppression (> 4 log 10 CFU/g feces); ↓ = mild to moderate suppression (2-4 log 10 CFU/g feces); ↑ = increase in number of organisms during therapy; - = no significant change; ∇ = decrease; + = positive result; Bifidus = Bifidobacterium spp.

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Table 1c. The Effects of Some Selected Antibiotics on GIT Microflora

Agent

Ciprofloxacin

Clindamycin

Doxycycline

Enoxacin

Erythromycin

Imipenem/ cilastatin

Lomefloxacin

Metronidazole

Moxalactam

Norfloxacin

Ofloxacin

Enterobacteria

↓↓

-

↓

↓↓

↓

↓↓

↓↓

-

↓↓

↓↓

↓↓

Enterococci

↓↓

↑

↓

-

↓

↓↓

-

-

↑

-

↓

Anaerobic bacteria

↓

↓↓

-

-

↓

↓↓

-

-

↓↓

-

-

Overgrowth of resistant strains

-

+

+

-

+

-

-

-

+

-

-

Days to normalization of flora (postadministration)

7

14

not stated

14

not stated

14

2129

not stated

14

1429

## Other

↑ in yeast colonization; no effect on Bifidus or Clostridia<sup>29,50</sup>

10% of drug excreted in bile; ∇ production of SCFAs; ∇ in Bifidus and Lactobacilli<sup>29,36,48,49</sup>

No effect on SCFA production<sup>29,36</sup>

↑ in Candida<sup>29</sup>

No significant change in Lactobacilli or Bifidus; ↑ in yeast colonization; ∇ production of SCFAs<sup>29,36,48</sup>

∇ in Lactobacilli and Bifidus<sup>29</sup>

No significant change in Lactobacilli, yeasts, Bifidus, or SCFA production<sup>29,36,48</sup>

∇ in Lactobacilli and Bifidus; ↑ in Candida and *C. difficile*<sup>29,48</sup>

∇ in Lactobacilli and Bifidus; ↑ in Candida<sup>29</sup>

## Impact on

An overview of some of the research investigating the effects of selected antibiotics on the GIT microflora. ↓↓ = strong suppression (> 4 log<sub>10</sub> CFU/g feces); ↓ = mild to moderate suppression (2-4 log<sub>10</sub> CFU/g feces); ↑ = increase in number of organisms during therapy; - = no significant change; ∇ = decrease; + = positive result; Bifidus = Bifidobacterium spp.

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The Impact of Antibiotics on GIT Microflora Antibiotic use is the most common and significant cause of major alterations in normal GIT microbiota.<sup>27</sup> The potential for an antimicrobial agent to influence gut microflora is related to its spectrum of activity, <sup>27</sup> pharmacokinetics, dosage,<sup>28</sup> and length of administration.<sup>29</sup> Regarding the spectrum of activity, an antimicrobial agent active against both gram-positive and -negative organisms will have a greater impact on the intestinal flora.<sup>27</sup>

Table 1d. The Effects of Some Selected Antibiotics on GIT Microflora

Agent

Pefloxacin

Phenoxymethylpenicillin

Piperacillin

Pivampicillin

Pivmecillinam

Talampicillin

Temocillin

Tetracycline

Ticarcillin/ Clavulanic acid

Tinidazole

Enterobacteria

↓↓

-

↓

-

↓↓

↑

↓↓

-

↓

-

Enterococci

-

-

↓

-

↑

-

-

-

↑

-

Anaerobic bacteria

-

-

↓

-

↓

--

-

-

↓

-

Overgrowth of resistant strains

-

-

-

+

+

+

-

+

-

-

Days to normalization of flora (postadministration)

not stated

14

not stated<sup>29</sup>

not stated

not stated

not stated<sup>29</sup>

not stated<sup>29</sup>

not stated

not stated

not stated

Other

No effect on Candida<sup>29</sup>

No significant change in Bifidus; larger doses ∇ Lactobacilli<sup>29,48,51</sup>

↑ in Candida; no change in Bifidus or Lactobacilli<sup>29,48,52</sup>

∇ in Lactobacilli and Bifidus<sup>29,48</sup>

↑ in Candida; ∇ in Bifidus and Lactobacilli<sup>29,48</sup>

∇ in Lactobacilli and Bifidus<sup>29</sup>

No significant change in Bifidus, Lactobacilli, or SCFAs<sup>29,48,53</sup>

Impact on

An overview of some of the research investigating the effects of selected antibiotics on the GIT microflora. ↓↓ = strong suppression (> 4 log<sub>10</sub> CFU/g feces); ↓ = mild to moderate suppression (2-4 log<sub>10</sub> CFU/g feces); ↑ = increase in number of organisms during therapy; - = no significant change; ∇ = decrease; + = positive result; Bifidus = Bifidobacterium spp.

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In terms of pharmacokinetics, the rate of intestinal absorption plays a fundamental role. Also important is whether the drug is excreted in its active form in bile or saliva. Both of these pharmacokinetic factors determine the drug's ultimate concentration in the intestinal lumen and, hence, the severity of the microfloral alteration.<sup>27</sup> In general, oral antimicrobials well absorbed in the small intestine will have minor impact on the colonic flora, whereas agents that are poorly absorbed can cause significant changes. Parenteral administration of antimicrobial agents is not free from these consequences, as some of these agents can be secreted in their active forms in bile, saliva, or from the intestinal mucosa, and result in considerable alterations in the colonic flora.<sup>30</sup> The dosage and length of administration of an antibiotic will also determine the magnitude of impact on the intestinal flora. In general, the greater the dosage and length of administration, the larger the impact on the microflora.<sup>29</sup> Tables 1a-1d provide an overview of research investigating the effects of specific antibiotics on GIT microflora. In general, the trials were conducted on healthy humans and involved only a single course of antibiotics. It is possible microfloral alterations induced by a particular antibiotic might be more severe in individuals with compromised health or who have been subjected to multiple courses of antibiotics. Recent epidemiological research has shown that individuals who had taken only one course of antibiotics had significantly lower serum concentrations of enterolactone up to 16 months post-antibiotic use compared to individuals who had remained antibiotic-free during the same time period ( $p < 0.05$ ). As serum concentrations of enterolactone are dependent on colonic conversion of plant lignans to enterolactone by the intestinal microflora (via beta-glycosidation), this study suggests infrequent antibiotic use has much longer-lasting effects on the microflora and its metabolic activities than was previously believed.<sup>31</sup> This negative association between serum enterolactone levels and antibiotic use has clinical importance

due to recent studies showing correlations between high serum enterolactone concentrations and protection from cardiovascular mortality<sup>32</sup> and breast cancer.<sup>33</sup> If an antimicrobial agent severely impacts the microflora, negative repercussions on host health can result, and include:

- Overgrowth of already-present microorganisms, such as fungi or *Clostridium difficile*.<sup>34</sup> Overgrowth of these organisms is a frequent cause of antibiotic-associated diarrhea, and overgrowth of *C. difficile* can develop into a severe life-threatening infection.<sup>35</sup>
- Decreased production of SCFAs, which can result in electrolyte imbalances and diarrhea.<sup>36</sup> Short-chain fatty acids play a vital role in electrolyte and water absorption in the colon.<sup>37</sup> Reduced production of SCFAs post-antibiotic use may be a causative factor in antibiotic-associated diarrhea.<sup>38</sup> Short-chain fatty acids also contribute to host health in other ways, such as improving colonic and hepatic blood flow,<sup>39</sup> increasing the solubility and absorption of calcium,<sup>40</sup> increasing the absorptive capacity of the small intestine,<sup>41</sup> and maintaining colonic mucosal integrity.<sup>42</sup>
- Increased susceptibility to intestinal pathogens due to the decrease in colonization resistance.<sup>43</sup> A decrease in colonization resistance after antibiotic administration has been observed in animal models. Such experiments have shown that disruption of normal microflora decreases the number of pathogens necessary to cause an infection and lengthens the time of infection.<sup>44</sup>
- Decreased therapeutic effect of some medicinal herbs and phytoestrogen-rich foods.<sup>31</sup> The activity of many medicinal herbs depends on bacterial enzymatic metabolism in the colon. Of the many enzymes produced by intestinal flora, bacterial beta-glycosidases probably play the most significant role, as many active herbal constituents are glycosides and are inert until the active aglycone is released via enzymatic hydrolysis.<sup>45</sup> Herbs such as willow bark (*Salix* spp.), senna (*Cassia senna*), rhubarb (*Rheum palmatum*), devil's claw (*Harpagophytum procumbens*), soy

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(*Glycine max*), and red clover (*Trifolium pratense*) would be essentially inactive without this colonic metabolism.<sup>45,46</sup> Based on the results of the above-described epidemiological study,<sup>31</sup> it can be inferred that antibiotic use interferes with microbial betaglycosidation in the GIT for a considerable period post-antibiotic administration, which could significantly impact the efficacy of many phytotherapeutic agents prescribed post-antibiotic use.

Hence, antimicrobial agents should be used sparingly and selected carefully in order to minimize the impact on GIT microflora.<sup>47</sup>

**The Effect of Stress on GIT Microflora** To determine whether psychological stress results in an altered gastrointestinal environment, Bailey and Coe investigated changes in indigenous GIT microflora in primates after maternal separation. GIT microflora was evaluated in 20 infant rhesus macaques ages 6-9 months who were separated from their mothers for the first time. All infant monkeys were found to have typical fecal bacterial concentrations at baseline. A brief increase in Lactobacilli shedding on the first day postseparation ( $p < 0.05$ ) was followed by a significant decrease in the concentration of Lactobacilli in the feces

( $p < 0.001$ ). An inverse relationship was also found between the fecal concentration of shed pathogens (*Shigella* spp. and *Campylobacter* spp.) and shed Lactobacilli ( $p = 0.07$ ). The study demonstrates that psychological stress can alter the integrity of indigenous microflora for several days.<sup>54</sup> Other authors have also theorized the Lactobacilli population responds to stress-induced changes in GIT physiology, such as inhibition of gastric acid release,<sup>55</sup> alterations in GIT motility,<sup>56</sup> or increased duodenal bicarbonate production.<sup>57</sup>

These changes may result in an intestinal environment less conducive to Lactobacilli survival, adherence, and replication. Alterations in GIT milieu may lead to detachment of Lactobacilli from the intestinal epithelium and subsequent passage through the GIT, thus resulting in decreased numbers of replicating Lactobacilli. This would explain the increased shedding of Lactobacilli found on the first day of stress, followed by a dramatic decrease in numbers of Lactobacilli over the next six days.<sup>54</sup> The effects of psychological stress on the intestinal environment have been studied in Soviet cosmonauts. In general, it was found that on return from space flight there was a decrease in fecal Bifidobacteria and Lactobacillus organisms (Table 2). These changes were attributed primarily to stress, although a diet low in fiber may also have contributed.<sup>58</sup>

The change in microflora observed by Lizko led to a subsequent decline in colonization resistance, which in turn resulted in increased numbers of potentially pathogenic organisms. It has been found that exposure to psychological stress results in a significant reduction in the production of mucin and a decreased presence of acidic mucopolysaccharides on the mucosal surface.<sup>58</sup> Since both mucin and acidic mucopolysaccharides are important for inhibiting adherence of pathogenic organisms to the gut mucosa, a decrease in either contributes significantly to successful colonization by pathogenic organisms.<sup>59</sup>

Table 2. Stress-associated Changes to GIT Microflora

During preparation

After short flight

After long flight

*L. acidophilus*

4.0

1.7

2.9

*L. casei*

3.5

0

0

*L. plantarum*

2.6

0.5

0

Changes in the Lactobacillus fecal flora in Soviet Cosmonauts (log/mL).<sup>58</sup>

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Lizko states that exposure to stress results in decreased production of immunoglobulin A (IgA). As IgA plays a vital role in the defense against pathogenic organisms by inhibiting bacterial adherence and promoting their elimination from the GIT, Lizko postulates that any decrease in IgA secretion would most likely increase intestinal colonization by potentially pathogenic microorganisms (PPMs).<sup>58</sup> A 1997 study assessed the effects of psychosocial stress on mucosal immunity, specifically the effect of emotional stress on secretory IgA (sIgA) levels.<sup>60</sup> The study was conducted on children ages 8-12 years (mean age 9.4 years). Ninety children were included in the trial – half of whom had a history of recurrent colds and flu, while the other half were healthy controls. The results demonstrated that stressful life events correlated with a decreased salivary ratio of sIgA to albumin. The ratio of sIgA to albumin controls for serum leakage of sIgA and is thought to give a clearer indication of mucosal immunity than total sIgA concentration. This result provides additional evidence of the likelihood of stress effectively decreasing mucosal immunity and, thus, diminishing intestinal colonization resistance. Other studies on college students have found sIgA concentrations decrease during or shortly after examinations.<sup>61</sup> Salivary concentrations of sIgA are inversely associated with norepinephrine concentrations, suggesting sympathetic nervous system activation suppresses the production and/or release of sIgA.<sup>60</sup> Thus, frequent suppression of mucosal immunity by the sympathetic nervous system during stressful experiences could increase colonization of the intestinal mucosa by PPMs. Holdeman et al studied factors that affect human fecal flora. They noted a 20-30 percent rise in the proportion of *Bacteroides fragilis* subsp. *thetaiotaomicron* in the feces of individuals in response to anger or fearful situations. When these situations were resolved, the concentration of these organisms in the feces decreased to normal levels.<sup>62</sup> This effect may be mediated via epinephrine, which has been shown to stimulate both intestinal motility and bile flow. As growth of *B. fragilis* subsp. *thetaiotaomicron* is enhanced by bile, this may partly explain the increased numbers of organisms in response to increased epinephrine release.<sup>63</sup> In vitro experiments conducted by Ernst and Lyte have demonstrated that several neurochemicals have the ability to directly enhance the growth of PPMs. The influence of the catecholamines norepinephrine, epinephrine, dopamine, and dopa were assessed on two strains of Enterobacteriaceae – *Yersinia enterocolitica* and *Escherichia coli*, and one strain of Pseudomonadaceae – *Pseudomonas aeruginosa*.<sup>64</sup> All three bacterial species are potential pathogens, with *Y. enterocolitica*<sup>65</sup> and *E. coli*<sup>66</sup> involved in GIT infections and *P. aeruginosa* in gastrointestinal, respiratory, and urinary tract infections.<sup>67</sup> The concentrations of catecholamines used in the experiment were equivalent to those found in plasma. The addition of norepinephrine, epinephrine, dopamine, and dopa to the cultures of *E. coli* resulted in increased growth when compared to non-catecholamine-supplemented control cultures. However, the largest increase in growth was observed with the addition of norepinephrine. Norepinephrine caused a large increase in growth of *Y. enterocolitica*, while both dopa and dopamine produced only small, but significant, increases in growth. Epinephrine demonstrated no effect. Norepinephrine also markedly increased the growth of *P. aeruginosa*, while the other catecholamines appeared to have no effect on this organism.<sup>64</sup> In vitro experiments performed by Lyte et al showed exposure of enterotoxigenic and enterohemorrhagic strains of *E. coli* to norepinephrine resulted in

increased growth and the expression of virulence factors, such as the K99 pilus adhesin, which is involved in the attachment and penetration of the bacterium into the host's intestinal mucosa. Growth of the enterohemorrhagic *E. coli* was also increased, as was its production of Shiga-like toxin I and Shiga-like toxin-II. The capability of norepinephrine to enhance both bacterial virulence-associated factors and growth was shown to be non-nutritional in nature – in other words, the bacteria did not use norepinephrine as a food; rather, the effect was via an unknown mechanism.<sup>68</sup> Additional experiments by Lyte et al demonstrated that upon exposure to norepinephrine, *E. coli* produces a growth hormone known as an

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“autoinducer of growth.”<sup>69</sup> This autoinducer showed a high degree of cross-species activity with other gram-negative bacteria, resulting in increased growth of other organisms. It was later found to stimulate 10- to 104-fold increases in the growth of 12 of 15 gram-negative microorganisms tested.<sup>70</sup> Exposure to stress has been documented to result in dramatic and sustained increases in catecholamine levels. This high concentration of catecholamines, and especially norepinephrine, may result in increased growth of PPMs in the intestines.<sup>61</sup> The GIT has abundant noradrenergic innervation and a high amount of norepinephrine is present throughout.<sup>71</sup> Studies conducted by Eisenhofer et al showed 45-50 percent of the total body production of norepinephrine occurs in the mesenteric organs.<sup>72,73</sup> Lyte suggests spillover of norepinephrine into the lumen of the intestinal tract undoubtedly occurs due to the concentration gradient present within the mesenteric organs.<sup>74</sup> Thus, there would be no requirement for an active transport system. This spillover effect has previously been demonstrated for serotonin following its release from gut enterochromaffin cells.<sup>75</sup> As such, the GIT represents an area in which neuroendocrine hormones like norepinephrine coexist with indigenous microflora.<sup>74</sup> Thus far, catecholamines have not been found to induce the growth of grampositive bacteria.<sup>70</sup> The effect of norepinephrine on gut flora was recently demonstrated in a murine model. The release of norepinephrine into the systemic circulation, caused by neurotoxin-induced noradrenergic neuron trauma, resulted in increased growth of gramnegative bacteria within the GIT. The total gramnegative population increased by 3 log units within the cecal wall and 5 log units within the cecal contents inside a 24-hour time period. The predominant species of gram-negative bacteria identified was *E. coli*.<sup>74</sup> To summarize, stress can induce significant alterations in GIT microflora, including a significant decrease in beneficial bacteria such as Lactobacilli and Bifidobacteria and an increase in PPMs such as *E. coli*. These changes may be caused by the growthenhancing effects of norepinephrine on gram-negative microorganisms or by stress-induced changes to GIT motility and secretions.

**Diet and Intestinal Microflora** The composition of the diet has been shown to have a significant impact on the content and metabolic activities of the human fecal flora.<sup>20</sup> Some diets promote the growth of beneficial microorganisms, while others promote microfloral activity that can be harmful to the host.

Sulfates Sulfur compounds, including sulfate and sulfite, have been shown to increase the growth of PPMs or increase production of potentially harmful bacterial products in the GIT. In the colon is a specialized class of gram-negative anaerobes known as sulfate-reducing bacteria (SRB). SRB include species belonging to the genera *Desulfotomaculum*, *Desulfovibrio*, *Desulfobulbus*, *Desulfobacter*, and *Desulfomonas*.<sup>76</sup> The principal genus, however, is *Desulfovibrio*, which accounts for 64-81 percent of all human colonic SRB. Sulfate-reducing bacteria utilize a process termed “dissimilatory sulfate reduction” to reduce sulfite and sulfate to sulfide.<sup>4</sup> The consequence of this process is the production of potentially toxic hydrogen sulfide, which can contribute to abdominal gas-distension.<sup>76</sup> Hydrogen sulfide can also damage colonic mucosa by inhibiting the oxidation of butyric acid, the primary fuel for enterocytes. Butyrate oxidation is essential for absorption of ions, mucus synthesis, and lipid synthesis for colonocyte membranes.<sup>77</sup> This inhibition of butyrate oxidation is characteristic of the defect observed in ulcerative colitis and leads to intracellular energy deficiency, as well as disruption of essential activities.<sup>4</sup> Sulfide has also been shown to cause a substantial increase in mucosal permeability, presumably due to the breakdown of the polymeric gel structure of mucin through the cleavage of disulfide bonds.<sup>4</sup> Sulfate-reducing bacteria are not present in all individuals and there appears to be considerable variation in SRB concentrations depending on geographical location, a variation hypothesized to be connected to dietary differences. Sulfate-reducing bacteria directly compete with methanogenic bacteria (MB) for vital substrates,

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such as hydrogen and acetate. In fact, methanogenesis and sulfate reduction appear to be mutually exclusive in the colon. In the presence of sufficient amounts of sulfate, SRB have been shown to outcompete MB for both hydrogen and acetate; whereas, under conditions of sulfate limitation the reverse occurs.<sup>78</sup> The amount of dietary sulfate that reaches the colon appears to be the primary factor in determining the growth of SRB. On the other hand, endogenous sources of sulfate (e.g., sulfated glycoproteins, chondroitin sulfate) appear to have little impact on SRB levels.<sup>79</sup> Sources of dietary sulfate include preservatives, dried fruits (if treated with sulfur dioxide), dehydrated vegetables, shellfish (fresh or frozen),<sup>80</sup> packaged fruit juices, baked goods,<sup>81</sup> white bread, and the majority of alcoholic beverages.<sup>6</sup> It also appears probable that ingestion of foods rich in sulfur-containing amino acids encourages both the growth of SRB and the production of sulfide in the large bowel.<sup>4</sup> Major amounts of sulfur-containing amino acids are found in cow’s milk, cheese, eggs, meat, and cruciferous vegetables. Consumption of large amounts of these foods may significantly increase sulfide production in the colon.<sup>77</sup> Research conducted in the 1960s found elimination of milk, cheese, and eggs from the diet of ulcerative colitis sufferers resulted in substantial therapeutic benefit, suggesting that reducing the intake of sulfur-containing amino acids decreases colonic production of sulfide.<sup>82</sup>

High Protein Diet Consumption of a high-protein diet can also increase the production of potentially harmful bacterial metabolites. It has been estimated that in individuals consuming a typical Western diet (containing ~ 100 g protein/day) as much as 12 g of

dietary protein per day can escape digestion in the upper GIT and reach the colon.<sup>83,84</sup> This is in addition to host-derived proteins, such as pancreatic and intestinal enzymes, mucins, glycoproteins, and sloughed epithelial cells.<sup>5</sup> Undigested protein is fermented by the colonic microflora with the resultant end-products of SCFAs, branched-chain fatty acids (e.g., isovalerate, isobutyrate, and 2-methylbutyrate), and potentially harmful metabolites – ammonia, amines, phenols, sulfide, and indoles.<sup>5,77,85</sup>

Ammonia has been shown to alter the morphology and intermediate metabolism, increase DNA synthesis, and reduce the lifespan of mucosal cells.<sup>6</sup> It is also considered to be more toxic to healthy mucosal cells than transformed cells and, thus, may potentially select for neoplastic growth.<sup>5</sup> Ammonia production and accumulation is also involved in the pathogenesis of portalsystemic encephalopathy.<sup>86</sup> Indoles, phenols, and amines have been implicated in schizophrenia<sup>87</sup> and migraines.<sup>88</sup> Indoles and phenols are also thought to act as co-carcinogens<sup>5</sup> and may play a role in the etiology of bladder and bowel cancer.<sup>83</sup> The production of these potentially toxic compounds has been found to be directly related to dietary protein intake,<sup>6</sup> a reduction of which can decrease production of harmful by-products.<sup>89</sup> The production of these potentially harmful by-products can also be attenuated by the consumption of diets high in fiber<sup>89</sup> and/or indigestible starch (both of which reduce intestinal pH).<sup>83</sup>

**Diets High In Animal Protein** In comparison to diets high in overall protein, diets especially high in animal protein have specific effects on intestinal microflora. While not appearing to dramatically alter the bacterial composition of the flora compared to control diets, ingestion of large amounts of animal protein does increase the activity of certain bacterial enzymes,<sup>90</sup> such as beta-glucuronidase, azoreductase, nitroreductase, and 7-alpha-hydroxysteroid dehydroxylase, in animals<sup>91,92</sup> and humans.<sup>93</sup> This can have important ramifications to the host, as any increase in activity of these enzymes will result in increased release of potentially toxic metabolites in the bowel. For instance, bacterial azoreductase can reduce the azo bond found in many synthetic food-coloring agents, releasing substituted phenyl and naphthyl amines, some of which are known to be potent carcinogens.<sup>90</sup> Another example is the action of the bacterial betaglucuronidases. Many xenobiotics are processed in the liver by a series of reactions that result in glucuronic acid conjugation. These glucuronides are then passed, via the biliary system, to the intestines. When these compounds reach the colon

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they can be hydrolyzed by beta-glucuronidase produced by the microflora, resulting in the release of the original xenobiotic, which then re-enters enterohepatic circulation and is recirculated several times before eventually being eliminated through the feces. If the original xenobiotic is mutagenic, carcinogenic, or otherwise toxic, this process can be detrimental to the host.<sup>94</sup> The nutrient calcium D-glucarate exerts its potentially beneficial effects by inhibiting beta-glucuronidase.

**High Simple Sugar/Refined Carbohydrate Diet** Kruis et al observed that diets high in simple sugars slow bowel transit time and increase fermentative bacterial activity and fecal concentrations of total and secondary bile acids in the colon.<sup>95</sup> A consequence of slower

bowel transit time may be an increased exposure to potentially toxic bowel contents.<sup>96</sup>  
 The mechanism by which high-sugar diets increase bowel transit time is not yet known.<sup>95</sup>

Table 3. The Effects of Various Diets on GIT Microflora

Microorganisms

Total anaerob es

Total aerob es

Bacteroides spp.

Enterococci

Bifidobact eria

Lactobacilli

Clostridia

Yeasts

American/ Mixed Western Diet

10.2a

7.5a

9.8a

5.5a

10.0a

7.3a

4.4a

-

American/ Seventh D ay Adventist Vegetarian D iet

-

-

11.7b

6.5b

8.1b

10.0b

8.6b

-

English/ Mixed Western Diet

10.1a

8.0a

9.8a 9.7a $\Psi$  5.8a 5.7a $\Psi$  9.8a 9.9a $\Psi$  6.5a 6.0a $\Psi$  5.0a 4.4a 1.3a $\Psi$

Japanese/ Japanese Diet

9.9a 11.4b 9.4a 9.8b 9.4a 10.1b 8.1a 8.4b 9.7a 8.2b 7.4a 5.7b 5.1a 9.7b

-

Japanese/ Mixed Western Diet

11.5b

9.6b

11.1b

8.4b

9.5b

4.0b

9.5b

-

## Ugandan/ Vegetarian Diet

9.3a

8.2a

8.2a 8.2aΨ 7.0a 7.0aΨ 9.3a 9.3aΨ 7.2a 7.2aΨ 4.6a 4.0a 3.1aΨ

## Indian/ Vegetarian Diet

9.7a

8.2a

9.2a

7.3a

9.6a

7.6a

5.0a

-

Effect of 'Western' vs. vegetarian or high carbohydrate diets on the human fecal flora (- = no data ; Ψ = a significant difference between groups; a = log<sub>10</sub> mean count/g wet weight of feces; b = log<sub>10</sub> mean count/g dry weight of feces.) From: Salminen S, Isolauri E, Onnela T. Gut flora in normal and altered states. *Chemother* 1995;41 (suppl 1):5-15.

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The increase in colonic fermentative activity noted in the Kruis study may not be directly associated with changes in microflora composition, but rather be caused by direct exposure of the colon to simple sugars. Refined sugars are metabolized quickly in the ascending colon; whereas, high-fiber foods, containing substantial amounts of insoluble fiber, are metabolized more slowly, releasing fermentation end-products (e.g., hydrogen gas and SCFAs) more gradually.<sup>97</sup> It is possible, however, that high sugar intake does cause alterations in the microflora. It has been observed that high sugar intakes increase bile output. Some species of intestinal bacteria utilize bile acids as food and, hence, any increase in their production will result in a competitive advantage for this group of bacteria.<sup>63</sup> The changes observed in bacterial fermentation in this study may or may not be related to changes in the species composition of the microflora. Since this was not adequately assessed in this study, the significance of these results requires further investigation. Other researchers have postulated that when intake of dietary carbohydrates is insufficient, increased fermentation of the protective layer of mucin may occur due to the limited quantity of carbon sources reaching the colon. This may compromise mucosal defense and lead to direct contact between colonic cells and bacterial products and antigens. This, in turn, may lead to inflammation and increased mucosal permeability. Such a situation may encourage the growth of potentially pathogenic bacteria and perpetuate the inflammatory response.<sup>98,99</sup> This theory, however, is yet to be supported by direct evidence.

**General Dietary Factors** The effect of the overall diet on the composition and metabolic activities of GIT microflora has been the subject of research since the late 1960s. It was initially believed that changing the content of the diet (in terms of meat, fat, carbohydrate, and fiber content) would dramatically alter the bacterial species composition of the colonic flora. However, when

the diets of various population groups consuming different diets were analyzed, the changes noted were not dramatic.<sup>93</sup> Only minor changes were noted among the groups, although these changes were considered to be caused by differences in diet.<sup>100</sup> Table 3 outlines results of several studies comparing the fecal flora of individuals consuming the typical Western diet (high in fat and meat) to that of individuals eating vegetarian and/or high complex-carbohydrate diets. In general, it appears populations consuming the typical Western diet have more fecal anaerobic bacteria, less Enterococci, and fewer yeasts than populations consuming a vegetarian or high complex-carbohydrate diet. Although one study found a significant difference between a mixed Western diet and a vegetarian diet, overall there appear to be relatively few trends. In spite of these findings, Gorbach argues that due to the sheer number of bacteria present in the stool (approximately 10<sup>11</sup> viable bacteria/g) and the enormous variety (around 500 anaerobic species, not to mention aerobic and facultative species), the classical method of quantifying flora is, at best, a crude approximation. Thus, these methods may be unable to differentiate changes due to variations in diet.<sup>90</sup> In an attempt to create a more sensitive method to detect changes in human microflora, Peltonen et al utilized gas-liquid chromatography (GLC) to analyze profiles of bacterial cellular fatty acids. This method measures bacterial cellular fatty acids present in the stool that accumulate to form a GLC fatty acid profile, with each peak in the profile representing relative amounts of a particular fatty acid in the stool. Similar bacterial compositions should yield similar fatty acid profiles, while distinctions can be quantified by the extent to which profiles differ from each other. The researchers utilized this technique to analyze the effects of a vegan, raw food diet on the intestinal microflora. The one-month diet consisted of a variety of sprouts, fermented vegetables, fruits, seaweed, nuts, and seeds. Differences in the GLC profile between the test and control groups were statistically significant ( $p < 0.05$ ), as were the differences in test group GLC profiles before and

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during the diet. No significant changes in the fecal flora could be detected in either group using the traditional isolation, identification, and enumeration bacteriological. While GLC may be a more sensitive method to determine changes in fecal flora, it cannot identify particular components of the flora.<sup>101</sup> Newer techniques such as fluorescence in situ hybridization (FISH) or polymerase chain reaction assays coupled with denaturing gel electrophoresis<sup>102</sup> are more sensitive to minor alterations in microflora and allow for bacterial identification that would otherwise be impossible to culture.<sup>103</sup> The use of these modern techniques in future diet studies will shed more light on this contentious area. Interestingly, recent research utilizing the FISH technique has indicated the majority of bacteria in the colon are not culturable and have yet to be described. This finding suggests how little is actually known about the composition of GIT microflora.<sup>104</sup> In summary, research has shown that consumption of foods rich in sulfur compounds, high in protein, and/or high in meat may produce detrimental effects on the host. These changes may be mediated through alterations in composition of the microflora or through increased production of bacterial metabolites. The impact of a high refined carbohydrate intake on the microflora has yet to be clearly elucidated. Similarly, the relationship between the overall



leading to tissue damage. Inflammatory bowel diseases can even involve areas far away from the gut, such as the extraintestinal manifestations involving the oral cavity with the onset of aphthous-like ulcers (ALU). Studies carried out on animal models have shown that intestinal dysbiosis may be related to the development of autoimmune diseases, even if the mechanisms involved are not yet well known. The aim of this paper is to verify the hypothesis that in inflammatory bowel diseases patients, aphthous-like ulcers are the result of the concomitance of intestinal dysbiosis and other events, e.g., the microtraumas, occurring in the oral mucosa, and that ex adjuvantibus therapy with probiotics can be employed to modify the natural course of the aphthous-like ulcers.

Keywords: microbiota; muco-microbiotic layer; dysbiosis; inflammatory bowel diseases; aphthouslike ulcers; probiotics

1. Introduction The human gut contains a high concentration of bacteria, collectively called microbiota. There are at least 1000 bacterial species (of which 150 to 500 only in the colon) with a density of  $9 \times 10^{13}$ – $14$ . Several roles, related to the regulation of the host's physiology, have been attributed to gut microbiota, including stimulation of the immune system, control of pathogenic bacterial proliferation, production of short-chain fatty acids, and fermentation of amino acids and saccharides [1,2]. A disequilibrium of the intestinal microbiota is called dysbiosis, and it causes an alteration of the intercellular tight junctions, allowing access of pathogens (and their toxins, in particular bacterial

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lipopolysaccharides) and stimulation of the mucosa-associated lymphatic tissue (MALT) with activation of the inflammatory cascade (leukocytes, cytokines,  $\text{TNF-}\alpha$ ), establishment of a chronic inflammation process (Figure 1) and, consequently, tissue damage [1].

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lipopolysaccharides) and stimulation of the mucosa-associated lymphatic tissue (MALT) with activation of the inflammatory cascade (leukocytes, cytokines,  $\text{TNF-}\alpha$ ), establishment of a chronic inflammation process (Figure 1) and, consequently, tissue damage [1].

Figure 1. The delicate equilibrium between eubiosis and dysbiosis in the bowels. Eubiosis is the condition in which saprophytic bacteria are present in the mucus-microbiotic layer of the bowel (either the small or the large one). Dysbiosis is a condition in which pathogenic bacteria (Pathogenic bacteria are represented with purple frame, non-pathogenic have a blue frame) predominate and cause changes in the intercellular tight junctions and, in turn, activation of the MALT, leading to tissue damage.

For this reason, dysbiosis has been implicated in the onset of several chronic autoimmune or inflammatory pathologies, including IBD (e.g., ulcerative colitis (UC) and Crohn's disease (CD)), metabolic diseases (e.g., obesity, type diabetes 2 and nonalcoholic fatty liver disease), autoimmune diseases (e.g., rheumatoid arthritis, allergies, and systemic lupus erythematosus), and other disorders (e.g., food intolerances and even colorectal cancer) [1,3]; to date, only few studies linked dysbiosis to primary or secondary immunological oral mucosal disorders [4–6]. The onset of extra-intestinal pathologies linked with dysbiosis is due to bacterial signals that affect the innate and adaptive immune system.

These signals also involve type 3 innate lymphoid cells, which contribute to the differentiation of T and B cells and induce the production of Th17 cells through secretion of IL-22. Moreover, it has been shown that the intestinal microbiota influences the accumulation of IgA-producing cells in the lumen, and that IgA diversity in the intestine is related to changes in microbiota composition. The intestinal microbiota also promotes the differentiation of naive CD4+ T cells into Th17, which act at the epithelial level to improve the integrity of the intestinal mucosal barrier. This suggests that a disruption at this level can cause changes in the intestinal barrier and the onset of various pathologies [7] affecting not only the bowel (e.g., UC), but also other organs (i.e., arthritis, uveitis, etc.).

1.1. Oral Manifestations in IBD Apart from the main symptoms related to the gastrointestinal involvement typical of IBD, these patients may present a broad spectrum of non-intestinal signs and symptoms known as extraintestinal manifestations (EIMs): joints, skin, eyes, the biliary tract and the oral mucosa are the most common sites involved [8].

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It is estimated that approximately one third of IBD patients may develop EIMs [9]; in particular, oral lesions may anticipate or accompany gastrointestinal illness: patients with IBD may present these manifestations years before the appearance of intestinal symptoms (5–10%), but EIMs are most commonly diagnosed after intestinal involvement has occurred [10]. EIMs can sometimes be confused with other extraintestinal complications

related to IBD due to malnutrition, chronic inflammation or side effects of drugs used to treat the disease itself [11]. The etiopathogenesis, classification, and natural history of muco-cutaneous disorders related to IBD have not yet been well defined: in general, oral lesions are found more often in patients with CD compared to UC, in children compared to adults and in males compared to females [8]. Differences may be found in the presentation of oral lesions between CD and UC: CD is characterized by both specific and nonspecific oral lesions, while only nonspecific ones are found in UC. In CD, oral lesions are defined as specific if the histopathologic data shows evidence of granulomas (similar to those observed endoscopically in the intestine); these are less common than nonspecific lesions (shown in Table 1) and they include: indurated tag-like lesions, cobblestoning, mucogingivitis, lip swelling, deep linear ulcerations and midline lip fissuring (Table 2).

Table 1. Clinical characteristics of nonspecific oral lesions in CD.

Nonspecific Oral Lesions	Clinical Presentation
Aphthous stomatitis	Shallow, round ulcers surrounded by an erythematous halo with a central fibrin membrane
Angular cheilitis	Erythema with/without painful fissures and sores at the corners of the mouth
Glossitis	Painful atrophy of the tongue
Pyostomatitis vegetans	

Small exophytic lesions covered with a vulnerable membrane, their cracking and confluence results in the characteristic sign of a "snail track" Oral Lichen/Oral Lichenoid reactions Associated to taste disturbances Gingivitis/Periodontitis Associated to a vitamin D deficiency

Table 2. Clinical characteristics of specific oral lesions in CD.

Specific Oral Lesions	Clinical Presentation
Indurated tag-like lesions (mucosal tags)	White reticular tags (labial and buccal vestibules, retromolar region)
Cobblestoning	Fissured and corrugated swollen mucosa with hyperplastic appearance (posterior buccal mucosa)
Mucogingivitis	Edematous, hyperplastic and granular gingiva (whole gingiva up to the mucogingival line)
Lip swelling	Associated to vertical fissures
Deep linear ulcerations	Associated to hyperplastic margins (vestibule)
Tongue and midline lip fissuring	Lip and tongue fissures

In particular, cobblestoning and tag-like lesions are considered pathognomonic for CD, but these are generally not associated with active intestinal disease [8]. Nonspecific oral lesions are found more often than the specific ones, and are usually associated with CD and UC; these include recurrent aphthae, angular cheilitis, pustular ulcerations, pyostomatitis vegetans, glossitis, lichen planus and nonspecific gingivitis [12]. Among the nonspecific findings, recurrent aphthae are the most known oral lesions associated with IBD; when the onset of aphthae is associated with systemic disorders, the term ALU is now

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considered preferable over the previously used RAS (recurrent aphthous stomatitis), since aphthae are considered a secondary manifestation and the different clinical courses of the two conditions require different management strategies [13]. ALU are reported to occur in up to 10% of UC and 25% of CD patients, and they may become more severe in active disease; however, their presence or absence does not correlate with disease activity [14]. Clinically, ALU are shallow, round or oval shaped lesions, granular on palpation; they are often painful, leading to negative effects on patients' daily activities [15]. Their onset is usually sudden and may be concurrent with a flare-

up of intestinal symptoms, or appear simultaneously with other EIMs [16]. Data from literature are conflicting in terms of the association between ALU and the pathological activity: some studies link the presence of oral lesions with the concomitant presence of intestinal symptoms, while other contradicting findings report no statistically significant difference [17,18]. Deficiencies caused by diet or poor absorption of an essential nutrient can cause anaemia and mineral and vitamin deficiency; in particular, vitamins B1, B2, B6, B12, iron, serum ferritin and folic acid deficiencies have been reported in the pathogenesis of oral ulcers, implicating their role in weakening the immune system [19]. Anaemia may also arise from chronic intestinal bleeding associated with iron deficiency, causing angular cheilitis and painful depapillation of the tongue; constant iron and zinc deficiencies may also be linked to erosive and crusty lesions on the lip commissures and perioral region [20]. Regarding IBD therapy, all of the currently used drug classes have been linked to alterations in the oral cavity due to their direct toxic effect on tissues and their indirect immunosuppressive effects [21].

**1.2. Morphology: Comparative Microscopic Anatomy of Oral and Intestinal Mucosa** All the organs of the alimentary canal have a common origin in the primitive digestive gut. The oral mucosa, that covers the entire oral cavity, consists of two layers: a stratified squamous epithelium and an underlying connective tissue (lamina propria) that includes blood and lymphatic vessels, as well as nerves and immune cells. However, the oral mucosa varies in structure, function and appearance in different regions of the cavity, and it is divided into lining, masticatory and specialized (gustative) mucosa [22,23], as detailed in Figure 2. Both the lining and masticatory oral mucosae may host aphthous lesions.

The wall of the small and large bowel is canonically divided into four layers: mucosa, submucosa, muscularis propria and serosa or adventitia. The mucosa is composed of epithelium, lamina propria and muscularis mucosae. A simple columnar epithelium covers the small and large bowel (Figure 2), although many regional differences are present [24–26]. The similarities between the oral and the intestinal mucosa include: (1) the presence of tight junctions between epithelial cells; and (2) the presence of a basement membrane between the epithelium and the lamina propria. The equilibrium between the epithelium and the lamina propria is very important for mucosal homeostasis, and both alterations in tight junctions and changes in basement membrane may result in dysfunction of the mucosal barrier, as seen in inflammatory bowel disease [27].

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oral mucosa, that covers the entire oral cavity, consists of two layers: a stratified squamous epithelium and an underlying connective tissue (lamina propria) that includes blood and lymphatic vessels, as well as nerves and immune cells. However, the oral mucosa varies in structure, function and appearance in different regions of the cavity, and it is divided into lining, masticatory and specialized (gustative) mucosa [22,23], as detailed in Figures 2. Both the lining and masticatory oral mucosae may host aphthous lesions.

Figure 2 Comparison between oral (both lining and masticatory, respectively subfigure a and b) and bowel (both small and large, respectively subfigure c and d) mucosae. Above: Original pictures, hematoxylin and eosin stainings; bar: 100 micra. Original magnifications: 100×. Below: drawings summarizing the main characteristics of these tissues. In detail, the

epithelium of oral mucosa is a stratified squamous epithelium, non-keratinized in the lining mucosa (subfigure a) and keratinized in the masticatory mucosa (subfigure b). It is divided into four layers: basal layer, prickle-cell layer, intermediate layer and superficial layer for lining mucosa; and basal layer, prickle-cell layer, granular layer and superficial (keratinizing) layer for masticatory mucosa. In both epithelia, the basal layer consists of cuboidal or columnar keratinocytes that are capable of division so as to maintain a constant epithelial population. Cells arising by division in the basal layers of the epithelium undergo a process of maturation as they are passively displaced toward the surface. In the non-keratinized squamous epithelium, the cytoplasm of intermediate cells does not contain keratin filaments. In keratinizing epithelium, the granulosum stratum is prominent and cells contain intracytoplasmic granules of keratohyaline. The epithelium of small bowel (c) covers the intestinal villi and the crypt compartments; it is columnar and composed of various cell types, such as absorptive cells, goblet cells and endocrine cells, in the villi, and stem cells and Paneth's cells, in the crypts. The epithelium of large bowel (d) covers glandular crypts; it is composed of a single layer of columnar cells and consist of absorptive cells that are responsible of water and ion transport, and goblet cells.

1.3. The Intestinal Microbiota and the Surrounding Mucous: The Fifth Layer of the Bowel Wall As stated before, the wall of the bowel is canonically divided into four layers by morphologists

through the observation of histological sections after processing them with reagents, including alcohols that remove mucus and other alcohol-soluble substances. However, in living subjects, the mucosal layer is characterized by the presence of a mix of symbiotic and pathogenic bacteria embedded in the mucus, produced by the epithelial cells. In this mucous matrix, apart from bacteria, are present a number of soluble substances and nanovesicles (i.e., exosomes, microvesicles and outer membrane vesicles), produced by both human cells and bacteria, that actively participate in the regulation of the homeostasis of the intestinal mucosa and, consequently, through lymphatic and hematic circulation, of virtually all of the organs [28–30]. Therefore, as already proposed [22], this mucus-microbiotic layer can be considered the real innermost layer of the intestinal wall.

The relevance of this hypothesis lies in the

fact that the understanding of the pathogenesis of human diseases derives from a precise knowledge of

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normal morphology, since many (if not all) pathologies derive from an alteration in cell differentiation that, in turn, generates tissutal changes and loss of organ function; thus most (if not all) treatment strategies should aim—when possible—to restore the normal

morphology of the organs. The aim of the present paper is to present a novel pathobiological hypothesis and, consequently, a non-invasive therapeutic method.

## 2. Pathobiology of ALU in IBD and Therapeutic Proposal

### 2.1. Pathobiology: A Focus on Dysbiosis

The persistence of dysbiosis causes a state of chronic inflammation linked to the activation of MALT and the release of inflammation mediators. This causes an onset of pathologies even in areas that are physically far away from the gut [1–3].

Intestinal epithelial cells represent the main communication barrier between the host environment and the microbiota, and also regulate the impact of the microbiota on the host immune function (Figure 3). For example, a healthy intestinal microbial flora promotes regulatory B (Breg) cell differentiation and IL-1b and IL-6 production, and controls inflammatory processes through Breg cells and IL-10 secretion.

Gut microbiota influences not only local, but also systemic immunity by bacterial metabolites (such as ligands of aryl hydrocarbon receptor and polyamines) and bacterial components, such as

polysaccharide A, that exhibit immunomodulatory action [31]; moreover, studies carried out on a

animal model have shown that intestinal dysbiosis may be related to the development of autoimmune diseases. Int. J. Mol. Sci. 2018, 19, x FOR PEER REVIEW 6 of 12 components, such as polysaccharide A, that exhibit immunomodulatory action [31]; moreover, studies carried out on animal models have shown that intestinal dysbiosis may be related to the development of autoimmune diseases.

Figure 3. Aphthous-like ulcers: histopathological features. Left: These images show some typical features of ALU: round or oval ulcer covered by a yellow-white fibromembrane with a peripheral erythematous halo; margins may appear indurated and elevated (A: ALU in the right side of the tongue; B: ALU in the posterior buccal mucosa; C: Four concomitant ALU in the anterior buccal mucosa). Right: The ulcerative lesion shows an increased angiogenesis and a mixed inflammatory infiltrate that consists of various leukocytes (lymphocytes, neutrophils, monocytes and histiocytes).

Mechanisms with which intestinal dysbiosis could generate autoimmune activation are not yet well understood. It is thought that they may be related to: - Alteration of Treg/Th17 due to dysregulated TLRs on antigen-presenting cells [27]; - Resistance to colonization, i.e., ability of the gut microbiota to limit the proliferation of external pathogens. It has been observed that in patients with autoimmune disorders (e.g., systemic lupus erythematosus), resistance to colonization was lower than in healthy controls; - Superantigens, derived from bacteria and viruses that have the ability to activate immune cells by simultaneously binding to the major proteins of the class II histocompatibility

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Right:

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- Superantigens, derived from bacteria and viruses that have the ability to activate immune cells by simultaneously binding to the major proteins of the class II histocompatibility complex (MHC II) present in the antigen-presenting cells and to specific receptors present in activated T cells [32];
- Alteration of host antigens and overproduction of autoantigens; in particular, microbiota induces modification of host proteins and creation of neoantigens [27,33];
- Mucosal responses to microbiota, i.e., inflammatory cytokines that activate nearby autoreactive cells [30];
- Molecular mimicry, i.e., cross-reactive antibody that recognizes shared epitopes of microbial and host tissue proteins, and activation of autoreactive T and B cells [29,34,35].

Thus, the hypothesis we formulate is that ALU is the result of the concomitance of intestinal dysbiosis (and consequent activation of the immune system) and other events, e.g., the microtraumas occurring (frequently and for various causes) in the oral mucosa.

Microtraumas can be considered as a stress factor for oral mucosa that induce overexpression, trafficking and surface mislocalization of intracellular proteins that may work, pathogenetically, as autoantigens. Heat shock proteins are an example of intracellular proteins that—after cell stress—may be mislocalized to cell surface by post-translational modifications that trigger unusual intracellular trafficking pathways; in addition, bacterial Hsp60 homologous, i.e., GroEL, can induce the formation of antibodies against it that can also cross-react against surface-exposed Hsp60, generating an autoimmune response by a molecular mimicry mechanism [36–39].

2.2. ALU Treatment: Can Probiotics Be Useful? In view of the above hypothesis, we suggest that an ex adjuvantibus therapy with probiotics could be able to modify the natural course of ALU (Figure 4).

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Figure 4. Probiotics as effective therapy for ALU. Restoration of eubiosis can dramatically contribute to remission of ALU by contrasting pathogenic phenomena. (Pathogenic bacteria are represented with purple frame, non-pathogenic with a blue frame; the yellow and green frame indicate the bacterial strains present after administration of probiotics)

We cannot yet precisely answer the question “how do probiotics work?” but some theories can be formulated. There are strong functional similarities between the gut and oral biofilms: it is reasonable to speculate that corresponding health-promoting events may occur in the oral cavity to those already reported in the gut. The oral cavity is a large reservoir of bacteria of >700 species and it is closely related to host health and disease [40,41]. In a recent study, it is demonstrated an association

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Probiotics as effective therapy for ALU. Restoration of eubiosis can dramatically contribute to remission of ALU by contrasting pathogenic phenomena. (Pathogenic bacteria are represented with purple frame, non-pathogenic with a blue frame; the yellow and green frame indicate the bacterial strains present after administration of probiotics).

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In the oral cavity, probiotics create a biofilm which matches with carcinogenic bacteria and periodontal pathogens modulating host immune response by strengthening the immune system [43]. There are local (direct) as well as systemic (indirect) events that occur by regulation of the immune response. The potential pathways could include [44]:

- Co-aggregation and growth inhibition;
- Bacteriocin and hydrogen peroxide production;
- Competitive exclusion through antagonistic activities on adhesion and nutrition;
- Immunomodulation.

There is an increasing body of evidence suggesting that perturbations of mucosal microbiota can modulate innate and adaptive immune responses, with inflammation arising upon reduction of the number of symbiont microorganisms and/or increase in the number of pathobiont microorganisms (commensal bacteria with pathogenic potential) [45]. Several immune mechanisms, implicated in the remission of ALU, by symbiont bacteria have been hypothesized, including induction of IL-10, suppression of TNF- $\alpha$  and IL-8, and modulation of Toll-like receptors [46].

This hypothesis has been reinforced by some studies that correlate the administration of probiotics to the improvement of autoimmune diseases. For example, it has been observed that in patients with rheumatoid arthritis, the administration of *Lactobacillus casei* increased the serum levels of IL-10 anti-inflammatory cytokine and decreased the levels of proinflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-12 [27].

3. Conclusions Intestinal dysbiosis causes a chronic inflammatory state and activation of the MALT in the gut, which leads to the onset of extraintestinal pathologies [1–3]. We

hypothesized that ALU could also be caused by intestinal dysbiosis, due to the immunological mechanisms involved in the pathogenesis of the disease [27] and the fact that there are several immune mechanisms implicated in the remission of ALU mediated by symbiont bacteria [36]. By comparing what happens in the intestine [47], we hypothesize that the administration of probiotics can increase the expression of tight junction protein ZO-1, both in terms of transcriptome and protein synthesis, with an improve intestinal barrier function. In fact, it was shown as a result of a chronic inflammatory state levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-23 stimulate the epithelial barrier breakdown, affecting in particular the expression of proteins forming the tight junctions.

This increase can be countered by administering specific probiotic strains including *Lactobacillus salivarius*, *Bifidobacterium lactis*, *Lactobacillus Plantarum* and *Lactobacillus fermentum* [48,49]. In addition to antagonistic act on proinflammatory cytokines, microbial metabolites directly promote the synthesis of the aforementioned tight junction proteins through activation of aryl hydrocarbon receptor, with subsequent activation of nuclear factor erythroid 2-related factor 2 (Nrf2) which has as a final result just the increased synthesis of ZO-1 with consequent strengthening of the epithelial barrier [50].

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Therefore, we proposed the use of probiotics as direct therapy for intestinal dysbiosis and as an indirect one for ALU. In particular, preliminary observations lead us to suggest that the therapy with probiotics should be started when the patient first starts to experience the ALU symptoms. Our hypothesis is that this therapy can reduce the duration of the disease by up to three days, through limiting the development of the lesion and favouring the re-epithelization of the lesioned oral cavity mucosa thanks to the molecular mechanisms discussed above (Figure 5). Int. J. Mol. Sci. 2018, 19, x FOR PEER REVIEW 9 of 12

Figure 5. Probiotics as effective therapy for ALU. Hypothesis: Restoration of eubiosis can dramatically contribute to remission of ALU by contrasting pathogenic phenomena. Experimental and clinical evidence on the use of probiotics for the treatment of oral aphthae are currently very limited, and the etiology and pathogenesis of ALU is currently unknown. So, we think that it would be opportune to carry out in depth studies of this phenomenon, taking into account that host genetics, nutritional deficiencies, and a number of systemic conditions have been recognized as systemic modulating factors of ALU [34,51]. Further studies are needed to establish which immunological mechanisms can be implicated in ALU pathogenesis and modulated by the administration of probiotics.

Funding:

## 21. What is molecular mimicry?

- a. When cells divide and grow into stem cells.
- b. When cells of the immune system mistakenly attack a sequence of peptides in “self” because it resembles the same sequence in a specific foreign antigen.
- c. When probiotics identify specific fibers to develop specific short chain fatty acids.
- d. When cells become damaged and become malignant.

### Molecular Mimicry as a Mechanism of Autoimmune Disease

Matthew F. Cusick & Jane E. Libbey & Robert S. Fujinami

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**Abstract** A variety of mechanisms have been suggested as the means by which infections can initiate and/or exacerbate autoimmune diseases. One mechanism is molecular mimicry, where a foreign antigen shares sequence or structural similarities with self-antigens. Molecular mimicry has typically been characterized on an antibody or T cell level. However, structural relatedness between pathogen and self does not account for T cell activation in a number of autoimmune diseases. A proposed mechanism that could have been misinterpreted for molecular mimicry is the expression of dual T cell receptors (TCR) on a single T cell. These T cells have dual reactivity to both foreign and self antigens leaving the host vulnerable to foreign insults capable of triggering an autoimmune response. In this review, we briefly discuss what is known about molecular mimicry followed by a discussion of the current understanding of dual TCRs. Finally, we discuss three mechanisms, including molecular mimicry, dual TCRs, and chimeric TCRs, by which dual reactivity of the T cell may play a role in autoimmune diseases.

**Keywords** Molecular mimicry. Autoimmune diseases. Dual T cell receptor. Virus infection. Immunopathology

Chronic autoimmune diseases are the by-product of the immune system recognizing self-antigens as foreign, which can lead to inflammation and destruction of specific tissues and organs (immunopathology) [1]. The impact of these diseases is global and heterogeneous with over 100 million

people afflicted with more than 80 different autoimmune diseases [2]. While the etiology of autoimmune diseases is not fully elucidated, the causes are likely based on a combination of hereditary and environmental factors [3]. Although host genetic background contributes to the induction of an immune response to self, epidemiological and molecular evidence implicates infectious agents (viral and bacterial) as the principal environmental insults responsible for the induction of autoimmune diseases (reviewed in [4–6]). Prolonged proinflammatory responses to infections have been associated with the initiation and exacerbation of autoimmune diseases (reviewed in [4, 7, 8]). Inflammation is facilitated by proinflammatory cytokines such as type I interferon (IFN), interleukin (IL)-1 $\beta$ , IL-12, IFN- $\gamma$ , IL-17, and tumor necrosis factor (TNF)- $\alpha$  (reviewed in [7, 9, 10]). However, these proinflammatory cytokines are critical for clearance of pathogens, suggesting that environmental factors are able to divert the immune response towards immunopathogenesis. Although a number of immune cells are responsible for secreting proinflammatory cytokines, the primary cell types implicated in a vast majority of autoimmune disorders are autoreactive B and T cells, or antibody recognition of self [11]. Although a number of viruses and bacteria have been linked to the initiation of certain

autoimmune diseases, identifying a particular virus or bacteria that is solely responsible for the induction of an autoimmune response is rare. This occurrence is due to the potential for multiple infections being involved in priming the immune system and other infections triggering disease, which could explain why no one viral infection has been conclusively linked to the development of immune-mediated autoimmune diseases [7]. However, there are a variety of examples of bacterial infections initiating and exacerbating autoimmune diseases. *Streptococcus pyogenes* is a gram-positive bacterium which

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Clinic Rev Allerg Immunol (2012) 42:102–111 DOI 10.1007/s12016-011-8294-7 causes group A streptococcal infection that is responsible for a number of diseases. The complications associated with *S. pyogenes* are rheumatic fever and glomerulonephritis. The infection causes the production of cross-reactive antibodies in response to the bacteria. Antibodies recognize the M protein (virulence factor) and the N-acetyl- $\beta$ -D-glucosamine (GlcNAc) of *S. pyogenes* and cross-react with myosin leading to heart damage (reviewed in [8, 12, 13]). Further evidence of molecular mimicry due to the production of cross-reactive antibody includes infection with gram-negative bacteria, such as *Klebsiella pneumoniae* and *Campylobacter jejuni*. Infection with *K. pneumoniae* or *C. jejuni* leads to the production of cross-reactive antibodies able to recognize the self-antigens histocompatibility leukocyte antigen (HLA)-B27 and gangliosides, which induce ankylosing spondylitis and Guillain-Barré syndrome, respectively (reviewed in [8, 14]). Examples of human autoimmune diseases with possible links with molecular mimicry are presented in Table 1. The immune system has a number of mechanisms that are able to detect foreign pathogens by utilizing the major histocompatibility complex (MHC). This locus encodes the HLA genes and a variety of immune response (Ir) genes, thereby shaping the immune system that protects against pathogens. There are two main types of HLA antigens, HLA class I and class II. The function of HLA class I molecules is to present viral peptides at the surface of an infected cell to a T cell receptor (TCR) on a CD8+ T cell. The activation of these CD8+ T cells leads to the killing of the virally infected cell. This role of HLA class I, the identification of cells that are infected, explains why all nucleated cells have the capacity to express these MHC molecules. HLA class II molecules, in comparison, are expressed almost exclusively on the surface of dendritic cells, B lymphocytes, macrophages, endothelial cells, and activated T cells. Functionally, the HLA class II molecules present peptides to the TCR on CD4+ helper T cells. The engagement of the TCR by the peptide-MHC complex is necessary for the activation of CD4+ and CD8+ T cells, thereby leading to an effective adaptive immune response against an invading pathogen [15]. CD4+ T cells are central mediators of the adaptive immune response including cytokine secretion and cellular and humoral defenses against a pathogen. The HLA locus is extremely polymorphic leading to a heterogeneous population ensuring propagation of a species against novel pathogens. Unfortunately, this genetic heterogeneity adds to the complexity of identifying HLA genes implicated in autoimmune diseases. In addition to its role in protection against pathogens, a second critical role of the MHC and Ir genes is to safeguard against self-reactivity by restriction of the immune response to self. In this regard, the immune system has developmental checkpoints for the maturation of a T cell. As a naïve T

cell expressing a pre-TCR migrates from the bone marrow to the thymus, rearrangement of  $\alpha$  and  $\beta$  TCR genes occurs and T cells that have either too high avidity or lack of recognition of self-antigens are selected against and subsequently programmed for cell death. This selection mechanism for generating mature  $\alpha\beta$  TCRs is named central tolerance. Further, peripheral mechanisms of tolerance are able to suppress autoreactive T cells through certain subsets of cells including regulatory T cells (Tregs) that are able to inhibit self-reactive immune cells in the periphery. Unfortunately, there are a variety of mechanisms including molecular mimicry, bystander activation, exposure of cryptic antigens, and superantigens by which pathogens can aid in the expression of an autoimmune disease [16–21]. Inflammation induced by exposure to a foreign antigen can lead to autoimmune diseases from cross-reactive epitopes (molecular mimicry). These epitopes are segments of foreign antigens which, when presented to either T or B cells in the context of the MHC, can activate CD4+ or CD8+ T cells. The induction of the immune response and subsequent proinflammatory cytokine release is critical for clearance of a virus or bacteria. However, a sustained proinflammatory response against specific host tissues can occur when there is sequence or structural homology between foreign antigens and selfantigens, termed molecular mimicry [18]. Although this concept has been associated with autoimmunity, there are instances where mimicry (cross-reactivity) provides protection for the host, termed heterologous immunity [22]. Cross-reactivity or mimicry between various strains of viruses or bacteria could help explain how protective immunity arises in certain individuals even in the absence of prior exposure to an emerging pathogen. This example of sequence homology in which molecular mimicry between viruses leads to protective immunity is in contrast to a pathogen mimicking host epitopes (reviewed in [11]).

#### Brief History of Molecular Mimicry

Over 30 years ago, molecular mimicry by either a virus [18] or bacteria [23] was hypothesized to initiate and exacerbate an autoimmune response through sequence or structural similarities with self-antigens. Currently, molecular mimicry is the prevailing hypothesis as to how viral antigens initiate and maintain autoimmune responses which lead to specific tissue damage [18]. Initial work by Fujinami, Oldstone, and colleagues identified mouse antibodies to measles virus and herpes simplex virus (HSV-1) obtained from antibody-secreting B cell clones [18]. These antibodies were reactive to both intermediate filaments of normal cells and the proteins of measles virus and HSV-1, *Clinic Rev Allerg Immunol* (2012) 42:102–111 103

Table 1 Examples of human autoimmune diseases with possible molecular mimicry as a mechanism

Human diseases	Target T cells/Ab	Human antigen mimicked	Organism	Reference(s)
Spondyloarthropathies (SpAs), ankylosing spondylitis, psoriatic arthritis, reactive arthritis and undifferentiated SpA				
Lumbar spine and sacroiliac joints	Abs	HLA-B27	<i>Klebsiella pneumoniae</i> , <i>Shigella</i> , <i>Chlamydia trachomatis</i> , and other gram-negative bacteria	[71–73]
Antiphospholipid syndrome	Fetal loss and thromboembolic phenomena	Abs $\beta$ 2-glycoprotein I	Bacteria, viruses, yeast, and tetanus toxin	[74]
Autoimmune chronic gastritis (AIG) (gastric atrophy, hypochloridria and pernicious anemia)	Stomach epithelium cells or parietal cell canaliculi	T cell/Abs H+, K +-ATPase, parietal cell canaliculi	<i>Helicobacter pylori</i>	[75]

Cogan's syndrome Eye and ear Abs SSA/Ro; (DEP-1/CD148); connexin 26 Reovirus III major core protein lambda 1 [76]  
 Autoimmune thrombocytopenic purpura Platelet Abs Platelet; platelet-associated immunoglobulin G (PAIgG) *Helicobacter pylori* [77]  
 Behçet's disease Eyes, skin, oral cavity, joints, genital system, CNS and blood vessels  
 T cell HSP60, HSP65, HSP70, alpha-tropomyosin, S-antigens  
 Mycobacterial HSP, *Plasmodium falciparum* [78–82]  
 Cardiomyopathy (myocarditis) Heart T cell / Abs Cardiac myosin Coxsackievirus, group A streptococci, chlamydia, or *Trypanosoma cruzi* [83]  
 Celiac sprue (celiac disease) Small intestine T cell Transglutaminase Gliadin (gluten), perinatal infections, adenovirus 12, hepatitis C virus (HCV) [84, 85]  
 Chagas disease Heart T cell Cardiac myosin *Trypanosoma cruzi* B13 protein [86, 87]  
 Chronic inflammatory demyelinating polyneuropathy Schwann cells Abs Monosialoganglioside GM2 Melanoma, *Campylobacter jejuni* [88, 89]  
 Crohn's disease Gastrointestinal tract T cell Unknown Gram-positive bacterial peptidoglycans [90]  
 Dermatomyositis (juvenile) Skin and muscle T cell Skeletal myosin *Streptococcus pyogenes* M5 protein [91] Essential mixed cryoglobulinemia B cell Abs IgG-Fc HCV [92] Guillain-Barré syndrome Gangliosides and peripheral nerve Abs Peripheral nerve *Campylobacter jejuni* [93]  
 Insulin independent diabetes (type I) Pancreas T cell Islet antigens (GAD65, proinsulin carboxypeptidase H) Coxsackie B virus, rubella, rotavirus, herpes, rhinovirus, hantavirus, flavivirus and retrovirus [94–96], (reviewed in [97]); [98–100]  
 Systemic lupus erythematosus Systemic Abs 60 kDa Ro Epstein-Barr virus (EBV nuclear antigen-1) [101] Multiple sclerosis Myelin T cell Myelin basic protein EBV, measles and HHV-6 [11, 35, 102]  
 Primary biliary cirrhosis Liver (intrahepatic bile ducts) Abs / Band T cell PDE2, GP210, human pyruvate dehydrogenase complex-E2 (PDC-E2), HLA-DR Gram-negative bacterium, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, cytomegalovirus, and *Haemophilus influenzae* [103–107]  
 Psoriasis Skin T cell Epidermal keratins *Streptococcus pyogenes* (streptococcal M protein) [108] Rheumatic fever Heart Abs / T cell Cardiac myosin M protein (major virulence factor of group A streptococci) and streptococcus carbohydrate epitope GlcNAc [12, 109–111]  
 Rasmussen's encephalitis CNS Abs Antigliamater receptor (GLUR3) Microorganisms [112, 113]  
 Acute disseminating encephalomyelitis CNS T cell Myelin basic protein Measles virus, rabies vaccine, HHV-6, coronavirus, influenza virus hemagglutinin, EBV, Semliki Forest virus [114, 115], reviewed in [116]  
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 thereby demonstrating a relatedness between host and viral antigens [18]. Further work by Fujinami and Oldstone used myelin basic protein (MBP), a nerve sheath protein containing an encephalitogenic T cell epitope in rabbits. The hepatitis B virus polymerase (HBVP) protein was found through computer analysis to share six consecutive amino acids with the encephalitogenic MBP epitope [16], and when rabbits were sensitized with either MBP or HBV peptides, the rabbit's tissue serum reacted against MBP. Further, rabbits sensitized with the HBVP peptide developed central nervous system (CNS) pathology similar to rabbits sensitized with whole MBP protein or the MBP peptide [16]. Importantly,

the rabbits sensitized with HBVP did not contract hepatitis but still developed encephalomyelitis and presented with a similar pathology as MBP-sensitized mice. These experiments were the first experimental demonstration of molecular mimicry, whereby a microbial peptide with similar amino acid sequences to the self-peptide was able to activate autoreactive T cells and subsequently cause specific tissue damage.

#### Relationship Between Molecular Mimicry and Autoimmune Diseases

Immune cells of the adaptive immune response are specifically activated, but the hallmark of autoimmunity is the dysregulation of the immune system, especially T and B cells recognizing self-antigens as foreign. The ability of T cells to evade central (thymic selection) and peripheral (Tregs) mechanisms of tolerance is evident by the large number of T cell-mediated human autoimmune diseases, such as type-1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (MS) [24–28]. Molecular mimicry has been implicated in the pathogenesis of many of these autoimmune diseases including MS, spondyloarthropathies, Graves' disease, and diabetes mellitus [16, 29, 30]. In the case of MS, it has been hypothesized that certain viruses, such as Epstein–Barr virus (EBV), share sequence homology with antigenic structures in the CNS [31]. Activation of an autoimmune response could be enhanced by a variety of other, albeit, non-mutually exclusive non-specific mechanisms including bystander activation and superantigens. The difference between other non-specific mechanisms that initiate autoimmunity and molecular mimicry is that microbial mimics specifically direct the immune response towards a tissue and/or organ. Originally, T cell recognition was postulated to be highly specific and cross-reactivity was thought to be a rare phenomenon. However, the structural requirements for peptide binding by MHC class II molecules that are presented to T cells were found to be based on amino

#### Table 1 (continued)

Human diseases	Target T cells/Ab	Human antigen mimicked	Organism	Reference(s)
Myasthenia gravis	CNS Abs	Acetylcholinereceptor, neurofilaments	Herpes simplex virus type 1g	pD [117]
Graft vs host disease	Solid organ transplant Abs	HLA-DR, CD13 (aminopeptidase N)	Human cytomegalovirus (hCMV)	[118, 119]
Herpes stromal keratitis	Eye T cell	Corneal tissue	Herpes simplex virus-type 1	[120]
Lyme arthritis	Joints Abs	Human leukocyte function-associated antigen-1 (hLFA-1)		
Borrelia burgdorferi	[121]	Sydenham's chorea	Brain Abs	$\beta$ -Tubulin, GlcNAc, calcium/calmodulin-dependent protein (CaM)
Goup	Astreptococcus	[122, 123]		
Autoimmune uveitis	Eye and pineal gland in the brain	T cell S-Antigen, interphotoreceptor binding protein (IRBP)	Viruses	[124]
Scleroderma	Endothelial cells	Abs NAG-2 (tetraspan novel antigen-2)	hCMV VUL94 protein	[125, 126]
Sjögren's syndrome	Systemic Abs	Ro 60 kD	Coxsackievirus	[127]
Stiff-person syndrome	Neurons and $\beta$ cells	T cell/Abs	GAD65	hCMV (pUL57) [128, 129]
Peptic/gastric ulcer	Gastric mucosa	Abs Gastric mucosa antigens (Lewis antigens)	Helicobacter pylori	[130]

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acid properties, and amino acids sharing similar chemical features were able to bind at the same MHC peptide binding groove, thereby demonstrating that binding motifs were degenerate with only a small sequence needed for TCR recognition [32–34]. An illustration of TCR degeneracy was shown by Wucherpfennig and Strominger [35] using chemically

related synthetic peptides mimicking the MBP(85–99) epitope that were incubated with human MBP(85–99)-specific T cell clones which were then tested for reactivity. Of the T cell clones that responded to the synthetic peptides, only eight of the 129 synthetic peptides were recognized by the T cell clones, and only one of the synthetic peptides that induced a response was clearly similar to MBP(85–99) [35, 36]. Therefore, these studies clearly demonstrate TCRs binding to a spectrum of specific peptides that is based upon structural relatedness, termed poly-specificity [37]. This flexibility exhibited by TCR binding and the existence of pathogens that share sequence or structural similarities with self-antigens could be one reason why investigators have been unable to conclusively associate a specific virus with autoimmune diseases, such as MS (reviewed in [4]). Linear sequence matches in amino acid motifs is not the only criteria for mimicry [32]. It has been hypothesized that self-reactive immune cells are primed by molecular mimicry and bystander activation, thereby sensitizing the immune cells and leading to a “fertile field” but no apparent disease. Subsequent environmental insults could induce these sensitized autoreactive cells to cause an autoimmune disease. Work from our laboratory demonstrated that recombinant viruses having molecular mimicry with self-CNS antigens were unable to initiate an autoimmune disease individually [38]. However, infected mice that were subsequently challenged, after viral clearance, with a non-specific immunologic insult developed disease [38]. Further, subsequent experiments showed that conventional inflammatory responses to specific pathogens were able to induce disease in animals primed with a molecular mimic to a CNS antigen [39]. Therefore, not only is the priming of the immune system necessary for an autoimmune disease but the milieu to which the primed immune cells are exposed is an important factor in initiating an autoimmune disease. Animal models of various autoimmune diseases have explored the role of molecular mimicry as a contributing factor (Table 2). The use of transgenic (tg) mice expressing virus proteins as transgenes in specific organs has been an important model for providing evidence for molecular mimicry. The expression of lymphocytic choriomeningitis virus (LCMV) viral antigens in pancreatic islet cells and the subsequent cross of this tg mouse with a TCR-tg mouse specific for LCMV glycoprotein resulted in an animal that only developed autoimmune disease if virally infected [40, 41]. These results demonstrated that “self”-reactive T cells are

present in the periphery and the immune cells appear to remain quiescent until an appropriate signal (viral infection) triggers the T cells to respond.

#### Dual TCR and How This Impacts Our Interpretation of Molecular Mimicry

There are a variety of non-mutually exclusive factors that lead to a fully activated T cell, such as the quantity of peptide–MHC presented on the surface of antigen presenting cells and TCR avidity. The interaction between the peptide–MHC and TCR is critical for the initiation of an adaptive immune response and clearance of a pathogen [15]. In order for T cells to reach maturity, the T cell goes through a number of developmental checkpoints leading to somatic recombination of various gene segments. The TCR  $\alpha$ - and  $\beta$ -chains are generated by V-D-J recombination, which leads to  $\alpha\beta$  TCRs expressed on the surface of T cells [42, 43]. Although it was believed that T cell signaling was mediated by a single antigen receptor, recent evidence demonstrates that T cells are capable of expressing functional dual V $\alpha$  TCRs at a frequency of approximately 30% in humans and 15% in mice; however, an accurate number of dual specific TCRs is lacking due to the limited availability of anti-V $\alpha$  monoclonal antibodies (mAbs) [44– 46]. Interestingly, in contrast to the high

frequency of dual expressing  $V\alpha$  T cells, only 1% of humans and 5–7% of mice express two  $\beta$ -chains due to allelic exclusion mechanisms, but the frequencies of dual  $V\beta$  TCRs have been found to be higher with age and in TCR-tg mice [47–49]. Expression of multiple TCR  $V\alpha$ s on the surface of a T cell is the result of simultaneous rearrangement of both TCR $\alpha$  loci during thymocyte development [50–52]. Further, TCR  $V\beta$ -chains preferentially bind to certain  $V\alpha$  chains leading to differential expression of chimeric TCRs on the surface of T cells [51, 53, 54]. Due to the heterogeneity of TCRs normally expressed in the periphery of humans and mice, TCR-tg mice have been used to track and determine the fate of T cells expressing dual TCRs. The use of TCR-tg mice has led to the identification of a potential role for dual TCRs in a variety of conditions including graft-versus-host disease, human immunodeficiency virus infection, inflammatory bowel disease, T cell leukemia, T cell lymphoma, and MS [55–61]. The expression of dual TCRs by the same T cell has been proposed to be a potential mechanism for autoimmune disease. Normally, high avidity self-reactive T cells are thymically depleted, but it has been hypothesized that the expression of a self-TCR on a T cell is lower when presented in the context of a second TCR, thereby providing a cover for high avidity self-TCRs from both central and peripheral tolerance. Blichfeldt et al. [62]

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demonstrated that dual TCRs, which have lower expression of each TCR on the surface of a T cell, needed higher concentrations of peptide, presented by MHC, to induce a similar T cell proliferative response compared to a single receptor T cell. A potential role of dual TCRs in autoimmunity is in the rescue of autoreactive T cells from thymic selection. For example, the double tg mouse for autoimmune diabetes, in which the mice express a TCR specific for peptide 111–119 of hemagglutinin (HA) (TCR-HA) under the control of the rat insulin promoter and develop spontaneous diabetes and insulinitis [63], were used to determine how T cells could escape tolerance mechanisms even if the antigen was ubiquitously expressed [64]. Low expressing TCR-HA coexpressing T cells were more effective at transferring diabetes than TCR-HA high dual TCRs, suggesting that the surface level expression of a dual TCR can be modulated by a second TCR expressed on the same T cell, thus “escape” of autoreactive T cells could be the first step in an autoimmune disease. The “trigger” of an autoimmune disease could be linked to environmental insults, such as viruses. A T cell co-expressing TCRs specific for a self-antigen and a foreign antigen could potentially allow for autoreactive T cells to be activated if the host is exposed to that foreign antigen. The activation of a subset of T cells could then lead to tolerance being broken and the initiation of an autoimmune disease if these T cells experienced a particular organ or tissue that expressed the self-antigen for the other TCR expressed at the surface of the T cell. In support of a role for dual TCRs in autoimmune diseases, work performed in our laboratory characterized autoreactive CD8<sup>+</sup> T cells isolated from the spleens of Theiler’s murine encephalomyelitis virus (TMEV) infected SJL/J mice [65]. In vitro assays testing CD8<sup>+</sup> T

cell killing activity found a population of CD8<sup>+</sup> T cells that killed uninfected syngeneic cells [65]. Adoptively transferring these TMEV-specific autoreactive CD8<sup>+</sup> T cells into non-infected SJL/J mice caused CNS pathology [65]. Further support for the importance of the mechanism by which viral infection could induce an autoimmune disease through dual TCR-expressing T cells was performed by Ji et al. [61] using MBP(79–87) TCR-tg mice [66]. Cytometric phenotyping, in vitro CD8<sup>+</sup> T cell killing assays, and adoptive transfer

experiments were used to track the expansion and killing capacity of V $\alpha$ 8V $\beta$ 8 MBP (79–87)-specific TCR and V $\alpha$ 8V $\beta$ 6-vaccinia virus-specific TCR. Infection of these tg mice with vaccinia virus induced autoimmune disease, thus demonstrating a virus triggering an autoimmune disease through dual TCR expressing T cells [61]. Although several tg TCR  $\beta$ -chains have been described on peripheral T cell [61, 67–70], there is no evidence that co-expression of dual TCRs leads to autoimmunity without the use of TCR-tg mice. As described above, current work in our laboratory has characterized TMEV-specific autoreactive CD8<sup>+</sup> T cell clones derived from a wild-type animal, and these autoreactive TMEV-specific T cell clones express dual TCRs (manuscript in preparation). Importantly, we were able to induce CNS pathology in naïve SJL/J mice by adoptively transferring the TMEV-specific clones. Although further work is needed in order to identify the self-antigen that activates these CD8<sup>+</sup> T cells, to our knowledge these results are the first demonstration of an autoimmune disease initiated by a dual expressing TCR characterized in the virus' natural host. Taken together, three possible mechanisms could explain how the dual reactivity of the TCR may play a role in autoimmune diseases (manuscript in preparation). The first mechanism is molecular mimicry, whereby the induction of

Table 2 Examples of murine models of autoimmune diseases where molecular mimicry is proposed as a mechanism

Human autoimmune disease	Mouse strain	Initiating agent(s)	Reference(s)
Behçet's disease	ICR mice	HSV type 1 (F strain) inoculation in ear lobe	[131]
Myocarditis	BALB/c	Mouse cytomegalovirus (MCMV)	[132]
Insulin-dependent diabetes (type I)	Tg mice	expressing LCMV protein in pancreas	Pichinde virus infection of mice [133]
Guillain-Barré syndrome	BALB/c	lipooligosaccharide of <i>Brucella melitensis</i>	[134]
Autoimmune hepatitis type 2	FVB	infection with recombinant adenovirus encoding human cytochrome CYP2D6	[135, 136]
Herpes stromal keratitis	C.AL-20	HSV-1	[120, 137]
Autoimmune uveitis	C3H/HeN	<i>Salmonella typhimurium</i>	[138, 139]
Sjögren's syndrome	C57BL/6; [B6] +/+; Fas-deficient B6-lpr/lpr; TNFR1-deficient B6; and TNFR1-deficient lpr/lpr	MCMV	[140]
Multiple sclerosis	SJL/J	C57BL/6 Theiler's murine encephalomyelitis virus	Semliki Forest virus infection [141] [142]

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an autoimmune response to self is due to a single TCR recognizing both a virus and a self-antigen. The second mechanism is the expression of dual TCRs on a single T cell, where one TCR is able to recognize a microbial antigen and the other TCR recognizes self. The third mechanism involves a T cell expressing chimeric TCRs generated from either a single V $\alpha$  combining with two different V $\beta$ s or a single V $\beta$  combining with two different V $\alpha$ s, resulting in a T cell with the potential of expressing two different chimeric TCRs specific for a self-antigen and a foreign antigen.

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22. A dental hygienist should teach patients to remove all dental plaque daily.

- a. True
- b. False

MICROBIAL ECOLOGY OF DENTAL PLAQUE AND ITS SIGNIFICANCE IN HEALTH AND DISEASE

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Abstract—Dental plaque forms naturally on teeth and is of benefit to the host by helping to prevent colonization by exogenous species. The bacterial composition of plaque remains relatively stable despite regular exposure to minor environmental perturbations. This stability (microbial homeostasis) is due in part to a dynamic balance of both synergistic and antagonistic microbial interactions. However, homeostasis can break down, leading to shifts in the balance of the microflora, thereby predisposing sites to disease. For example, the frequent exposure of plaque to low pH leads to inhibition of acid-sensitive species and the selection of organisms with an aciduric physiology, such as mutans streptococci and lactobacilli. Similarly, plaque accumulation around the gingival margin leads to an inflammatory host response and an increased flow of gingival crevicular fluid. The subgingival microflora shifts from being mainly Grampositive to being comprised of increased levels of obligately anaerobic, asaccharolytic Gram-negative organisms. It is proposed that disease can be prevented or treated not only by targeting the putative pathogens but also by interfering with the processes that drive the breakdown in homeostasis. Thus, the rate of acid production following sugar intake could be reduced by fluoride, alternative sweeteners, and low concentrations of antimicrobial agents, while oxygenating or redox agents could raise the Eh of periodontal pockets and prevent the growth and metabolism of obligately anaerobic species. These views have been incorporated into a modified hypothesis (the "ecological plaque hypothesis") to explain the relationship between the plaque microflora and the host in health and disease, and to identify new strategies for disease prevention.

This manuscript was presented at a Symposium entitled "Mechanisms and Agents in Preventive Dentistry", held October 28-November 7, 1992, in Chester, England, under the auspices of the Council of Europe Research Group on Surface and Colloid Phenomena. The mouth is similar to other sections of the digestive tract in having a resident microflora that develops naturally, and which has a characteristic composition. Owing to differences in local environmental conditions, however, the microflora of mucosal surfaces differs in composition from that of dental plaque. For similar reasons, the plaque microflora varies in composition at distinct anatomical sites on the tooth—for example, in fissures, on approximal surfaces, and in the gingival crevice. The resident microflora of a site is of benefit to the host by acting as part of the host defenses by preventing colonization by exogenous (and often pathogenic) micro-organisms ("colonization resistance"; van der Waaij et al., 1971). The early colonizers of the tooth surface include members of the genera *Streptococcus*, *Actinomyces*, *Haemophilus*, *Neisseria*, and *Veillonella* (Liljemark et al., 1986; Nyvad and Kilian, 1987). These bacteria adhere to the acquired enamel pellicle by specific and non-specific molecular interactions between adhesins on the cell and receptors on the

surface (Gibbons, 1989; Busscher et al, 1992). Once established, the microflora at a site remains relatively stable over time despite regular minor perturbations to the oral environment (Marsh, 1989). This stability (termed "microbial homeostasis") stems not from any metabolic indifference among the components of the microflora, but rather results from a dynamic balance of microbial interactions, including both synergism and antagonism (Sanders and Sanders, 1984).

**MECHANISMS INVOLVED IN MAINTAINING MICROBIAL HOMEOSTASIS** It has been proposed that the ability to maintain homeostasis within a microbial community increases with its species diversity (Alexander, 1971). In dental plaque, the diversity of the microflora is enhanced by the development of food chains between bacterial species, and their use of complementary metabolic strategies for the catabolism of endogenous nutrients, such as glycoproteins and proteins. Individual species possess different but overlapping patterns of enzyme activity so that certain mixed cultures of oral bacteria can synergistically degrade complex host molecules (van der Hoeven and Camp, 1991). Several food chains have been recognized among plaque bacteria (Mikx and van Campen, 1982; Grenier and Mayrand, 1986), such as the utilization of lactic acid by *Veillonella* spp. and succinate by spirochetes. Antagonism is also a major mechanism in maintaining microbial homeostasis in plaque. Bacteriocins and bacteriocinlike substances are produced by many genera of oral bacteria (James and Tagg, 1988; Marsh, 1989). The precise benefit of bacteriocins is not known for certainty, but animal studies have shown that their production can confer an ecological advantage on an organism during colonization (van der Hoeven and

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TABLE 1 EFFECTS OF GLUCOSE PULSES, LOW pH, AND FLUORIDE ON THE STABILITY OF A MIXED CULTURE OF NINE ORAL BACTERIAa

Bacterium *S. gordonii*

*S. oralis* *S. mutans* *A. viscosus* *L. casei* *N. subflava* *V. dispar* *P. intermedia* *F. nucleatum* Final pH

Pre-pulsing 28.3 15.2

0.3 0.1 0.1 0.1 9.8 31.0 15.2 7.0

Percentage

With pH control (pH7) 25.0 16.9

1.0 13.1 0.2 0.01 28.7 5.6 9.5 7.0

Viable Count

After 10

Without pH control 0.2

1.3

18.9 2.3 36.1 NDb 41.4  $6 \times 10^4$   $2 \times 10^5$  3.83

glucose pulses: Without pH control, with NaF 0.002

4.6 0.2 0.4 36.5  $2 \times 10^5$  57.8 0.5 0.2

4.49 The mixed culture was pulsed on 10 consecutive days to give 28 mmol/L glucose, with or without 1 mmol/L NaF. The pH was either maintained automatically throughout at pH  $7.0 \pm 0.1$  or was allowed to fall for six h following each pulse before being returned to pH

7.0 for 18 h prior to the next pulse. b ND = not detected. (Data from Bradshaw et al, 1989a, 1990)

Rogers, 1979). Other inhibitory factors produced by plaque bacteria include organic acids (Donoghue and Tyler, 1975), H<sub>2</sub>O<sub>2</sub> (Holmberg and Hallander, 1973), and enzymes (Baba, 1986). The production of such inhibitory substances might also be a major factor in determining the composition of the plaque microflora. It was found that subgingival plaque samples from healthy subjects contained organisms that could inhibit the growth of several periodontopathogens (Hillman and Socransky, 1989). In contrast, plaque from sites with localized juvenile periodontitis (LJP) or refractory periodontitis invariably lacked organisms producing inhibitors. Subsequent studies identified some of the antagonistic bacteria as *Streptococcus sanguis*, and the inhibitor as H<sub>2</sub>O<sub>2</sub> (Hillman and Socransky, 1989). Such interactions can also contribute to colonization resistance. *S. salivarius* can produce "enocin" with activity against Lancefield Group A streptococci (Sanders and Sanders, 1982) and may prevent colonization of this pathogen in the mouth in a manner similar to that proposed for the pharynx.

**PLAQUE MICROFLORA AND DISEASE** Plaque accumulates preferentially at stagnant or retentive sites, unless removed by diligent oral hygiene. As plaque mass increases, saliva is less able to penetrate plaque and protect enamel. Microbial homeostasis can break down, and major shifts in the composition of the microflora can occur. For example, the frequent consumption of fermentable dietary carbohydrates is associated with an increased risk of dental caries (Loesche, 1986). Such diets lead to a rise in the proportions of mutans streptococci and lactobacilli, with a concomitant fall in levels of other streptococci, especially members of the *Streptococcus oralis* group, which include *S. sanguis*, *S. oralis*, and *S. mitis* (de Stoppelaar et al, 1970; Dennis<sup>a</sup> et al, 1975; Staat et al, 1975; Minahefa et al, 1985). The metabolism of plaque also changes from a heterofermentative pattern to one in which sugars are converted primarily to lactic acid. Gingivitis is associated with the accumulation of plaque around the gingival margin. The host mounts an inflammatory response to this microbial challenge, and the flow of gingival crevicular fluid (GCF) is increased. The composition of subgingival plaque shifts away from a streptococci-dominated microflora (Slots, 1977) to one with higher levels of *Actinomyces* spp. and an increase in capnophilic and obligately anaerobic bacteria such as *Capnocytophaga*, *Fusobacterium*, and *Prevotella* species (Savitt and Socransky, 1984; Moore et al, 1987). Gingivitis may lead to more advanced forms of periodontal disease, in which the microflora can become even more diverse. Depending on the type of disease, bacteria belonging to the genera *Actinobacillus*, *Campylobacter*, *Selenomonas*, *Treponema*, and *Wolinella* may be isolated. Tissue damage can result directly from the activity of the subgingival microflora and indirectly from the release of lysosomal enzymes during phagocytosis or to the production of cytokines that stimulate resident connective tissue cells to release metalloproteinases (Reynolds, 1994).

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TABLE 2

COMPONENTS OF GINGIVAL CREVICULAR FLUID  
THAT MIGHT AFFECT THE COMPOSITION  
OF THE SUBGINGIVAL MICROFLORA Host Defenses

IgG IgA IgM  
 Complement  
 B and T lymphocytes Neutrophils Macrophages  
 Novel Nutrients Hemin, iron Albumin a-2-globulin Transferrin  
 Hemopexin  
 Hormones Haptoglobin Hemoglobin Proteins, glycoproteins

TABLE 3

PREVENTION STRATEGIES AND THE ECOLOGICAL PLAQUE HYPOTHESIS

1. Reduced sugar/low pH challenge (a) Fluoride (b) Sugar substitutes (c) Stimulation of saliva flow (d) Antimicrobial agents (sub-MIC) 2. Altered subgingival environment (a) Oxygenating or redox agents (b) Anti-inflammatory agents (c) Antimicrobial agents (sub-MIC) 3. Replacement therapy (a) Pre-emptive colonization (b) Competitive displacement

POTENTIAL FACTORS DISRUPTING MICROBIAL HOMEOSTASIS IN DENTAL PLAQUE

Observations from clinical and laboratory studies have enabled potential factors to be recognized that may disrupt microbial homeostasis in plaque. Several in vitro model systems have been devised to study interactions among the oral microflora, the environment, and the host. Some have tried to simulate important physical aspects of the oral cavity by incorporating surfaces for biofilm formation (the "artificial mouth"; see Tatevossian, 1991), while an alternative approach has been to exploit the unique advantages of the chemostat to grow mixed cultures of oral bacteria under a range of defined but controllable conditions (Marsh, 1993). Individual parameters can be varied independently in the chemostat, and their effects on the composition and metabolism of the culture can be determined, so that cause-and-effect relationships can be established. Several factors that may be responsible for the transition of the oral microflora from having a commensal to a pathogenic relationship with the host have been identified by means of continuous-culture techniques. Mixed-culture chemostat studies have been performed to distinguish whether the increases in mutans streptococci and lactobacilli following repeated sugar intake are due to differences in the ability of oral bacteria to (a) transport and catabolize sugars, or to (b) tolerate and grow in the low-pH environment so generated. A system for growing nine oral bacteria, stably and reproducibly, in a chemostat at constant temperature (37°C) and pH (7.0 ± 0.1) in a habitat-simulating medium has been developed (see Marsh, 1993). The effect on the balance of the mixed culture of pulsing on 10 consecutive days with glucose, either with or without pH control, was determined (Table 1). It was found that low pH rather than the availability of carbohydrate per se was the factor driving the selection of potentially cariogenic species. This selection was at the expense of acid-sensitive species, some of which are associated more with oral health (Bradshaw et al., 1989a). The

experiment was repeated to determine if there was a "critical pH" for this breakdown in homeostasis to occur. The culture was again pulsed with glucose in three replicate experiments but in which the pH was allowed to fall only to fixed values of pH 5.5, 5.0, or 4.5, respectively. The microbial community was disrupted irreversibly only when the pH fell regularly below pH 5.0 (Bradshaw et al., 1989b), and the predominant species always became *Streptococcus mutans*, *Lactobacillus casei*, and *Veillonella dispar*. These three species have been associated with nursing caries (Milnes and Bowden, 1985) and progressing caries (Boyar and Bowden, 1985) in humans. Pure culture studies have also shown that the growth of these three species is less sensitive to low pH than other oral

bacteria (Harper and Loesche, 1984; Bradshaw et al., 1989a). Furthermore, mouthrinsing with acidic buffers (pH 3.9) was found to increase the proportions of mutans streptococci in human fissure plaque (Svanberg, 1980). Collectively, these findings show that the selection of cariogenic species following regular sugar consumption is likely to be a consequence of their aciduric physiology, which enables them to compete successfully at low pH. In periodontal diseases, the redox potential of pockets is lower than that at healthy sites (Kenney and Ash, 1969). The inflammatory host response also leads to increased secretion of GCF and a small rise in local pH from just below neutrality in health to around pH 7.5 during disease (Eggert et al., 1991). GCF not only delivers components of the host defenses but also provides a continuous supply of proteins, glycoproteins, and co-factors that can act as novel nutrients for bacteria, especially asaccharolytic and obligately anaerobic species (Table 2). In an early longitudinal clinical study, black-pigmented anaerobes increased from 0.01 to 0.2% of the subgingival flora when gingivitis progressed to a bleeding stage (Loesche and Syed, 1978). This is noteworthy, because these organisms require hemin for growth, and this co-factor can be derived from the

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ECOLOGICAL PLAQUE HYPOTHESIS

AND PREVENTION OF CARIES

FERMENTABLE SUGAR REMINERALIZATION

DEMINERALIZATION

Fig. 1—The ecological plaque hypothesis and the etiology of caries. The diagram depicts a dynamic relationship whereby an environmental change in plaque (low pH) produces a shift in the balance of the resident microflora, thereby favoring demoralization. Caries could be prevented not only by inhibiting the putative pathogens but also by interfering with the environmental change driving the ecological shift. MS = mutans streptococci.

ECOLOGICAL PLAQUE HYPOTHESIS AND

PREVENTION OF PERIODONTAL DISEASE

Plaque Reduction

Plaque Accumuli

Fig. 2—The ecological plaque hypothesis and the 1 etiology of periodontal diseases. The diagram depicts the dynamic relationship whereby the inflammatory response results in an environmental change, subgingivally, which produces a shift in the balance of the resident microflora. Such a shift predisposes a site to disease. Disease could be prevented not only by inhibiting the periodontopathogens directly but also by interfering with the factors driving the transition.

degradation of host molecules in GCF. Similarly, it was reported recently that some species that predominate in periodontitis, but which are not detectable in the healthy gingiva, can be found as a small proportion of the microflora in gingivitis (Moore et al., 1987). This also suggests that environmental conditions which develop during gingivitis {e.g., bleeding, increased GCF flow) may favor the growth of species implicated in periodontitis. The possible effect of GCF on the stability of the subgingival microflora has been studied in the laboratory by repeated passaging of plaque through human serum (used as a substitute for GCF) (ter Steeg et al., 1981) or by the prolonged continuous culture of plaque on serum (ter Steeg et al., 1988). Both experimental approaches resulted in the enrichment of species

implicated in periodontal disease (e.g., anaerobic streptococci, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Treponema denticola*) that were present in the inoculum at levels too low to be detected. Likewise, the effect of the clinically-observed rise in pH during inflammation on the proportions of three black-pigmented anaerobes has been studied in mixed-continuous culture. At or below pH 7.0, the culture was dominated by *Prevotella melaninogenica*. As the pH was increased to pH 7.25, *P. intermedia* became predominant, whereas at pH 7.5 and above, the culture was comprised of >99% *Porphyromonas gingivalis* (McDermid et al., 1990). These studies have demonstrated the significant influence an altered supply of nutrients and even a small change in local pH can have in determining the balance of the microflora.

**IMPLICATIONS FOR THE ETIOLOGY OF CARIES AND PERIODONTAL DISEASES** There are two main schools of thought on the role of plaque bacteria in disease. The "specific plaque hypothesis" (Loesche, 1976) proposes that, out of the diverse collection of microorganisms that constitute the resident plaque microflora, only a very limited number are actively involved in causing disease. Problems can arise with this hypothesis, however, when attempting to explain those occasions when either disease is diagnosed in the apparent absence of the putative pathogens, or when pathogens are present at sites with no evidence of disease. When sensitive detection methods are used, mutans streptococci can be found quite commonly in plaque, albeit in low numbers (Bratthall, 1991), as can periodontopathogens such as *F. nucleatum*, *P. intermedia*, and, on occasion, *A. actinomycetemcomitans* (Ashley et al., 1988; Frisken et al., 1987; Zimmer et al., 1991). In contrast, the "non-specific plaque hypothesis" purports that many of the heterogeneous mixture of organisms in plaque could play a role in disease, and that disease is a result of the overall interaction of the plaque microflora with the host (Theilade, 1986). Some of the arguments surrounding these hypotheses may be, in part, about semantics (e.g., the definition of "specific" or "non-specific"), since plaque-mediated diseases, while not necessarily having a totally specific etiology, do show evidence of specificity. Consequently, a modified hypothesis (the "ecological plaque hypothesis") was proposed recently (Marsh, 1991) in an attempt to unify some of these clinical and laboratory observations. In this hypothesis, it is proposed that a change in a key environmental factor (or factors) will trigger a shift in the balance of the resident plaque microflora, and this might predispose a site to disease (Figs. 1 and 2). The occurrence of potentially pathogenic species as minor members of the resident plaque microflora would be consistent with this proposal. Under the conditions that prevail in health, these organisms would be only weakly competitive and may also be suppressed by inter-microbial antagonism, so that they would comprise only a small percentage of the plaque microflora and would not be significant clinically. Microbial specificity in disease would be due to the fact that only certain species are competitive

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under the new (changed) environmental conditions. It is a basic tenet of microbial ecology that a major change to an ecosystem produces a corresponding disturbance to the stability of the resident microbial community (Brock, 1966; Alexander, 1971; Fletcher et al., 1987). Examples of this have been reported in ecosystems as diverse as soil (Heal and Ineson, 1984), skin (Noble and Pitcher, 1978), and water (Pomeroy, 1984). In subjects with a

conventional low-sugar diet, the composition of the plaque microflora would be stable, and only small amounts of acid would be produced at main-meal times. In these circumstances, the processes of de- and remineralization would be in equilibrium. If the frequency of fermentable dietary carbohydrate intake were to increase, however, plaque would spend more time at low pH (Loesche, 1986). Such conditions would favor the proliferation of mutans streptococci and lactobacilli at the expense of more acid-tolerant species, and this would tip the equilibrium toward demineralization (Fig. 1). Factors reducing the flow of saliva (e.g., xerostomia) would lead to similar shifts in the microflora. Greater numbers of mutans streptococci and lactobacilli would lead to even faster rates of acid production from sugars, enhancing demineralization still further, while the elevated levels of lactic acid in plaque would also select for *Veillonella* spp., as has been reported in the aforementioned clinical studies. Acid-sensitive species, such as members of the *S. oralis* group (e.g., *S. sanguis*, *S. oralis*, and *S. mitis*), would decline in proportion, thereby accounting for the widely reported inverse relationship between *S. sanguis* and mutans streptococci seen in plaque. Other bacteria could also produce significant amounts of acid under similar conditions, at slower rates (van Houte, 1992), but nevertheless providing an explanation for demineralization in the absence of mutans streptococci. Likewise, in periodontal diseases, the changes in nutrient profile of the gingival crevice due to GCF secretion will lead to increased plaque biomass, the metabolism of which will lower the redox potential of the site and raise the pH. These changes will tend to enrich the previously low levels of obligately anaerobic and often asaccharolytic Gram-negative bacteria, and so fuel the inflammatory response (Fig. 2). Other predisposing factors might include the state of the host defenses, so that leukotoxin-producing strains of *A. actinomycetemcomitans* might exploit the pocket environment in individuals with neutrophil deficiencies and gain a competitive advantage (Genco and Slots, 1984). These sequences of events can go some way toward explaining the lack of total specificity in the microbial etiology of dental caries and periodontal diseases, and account for the pattern of bacterial succession often seen during disease progression in clinical studies.

**IMPLICATIONS FOR DISEASE PREVENTION** Implicit in the ecological plaque hypothesis is the possibility that disease might be prevented, not only by inhibiting the putative pathogen(s), but also by interfering with the factors responsible for the transition of the plaque microflora from having a commensal to a pathogenic relationship with the host (Marsh, 1991). A consideration of the principles behind the ecological plaque hypothesis can lead to the identification of new strategies to prevent disease, as well as enabling existing approaches to be seen from a fresh perspective (Table 3). These strategies will now be discussed in terms of their ecological implications.

**Strategies to prevent dental caries** One of the primary mechanisms by which microbial homeostasis in the mouth can be disrupted is by the repeated exposure of plaque to low pH following the frequent intake of fermentable dietary carbohydrates (Loesche, 1986). Consistent with the prevention of disease under the ecological plaque hypothesis would be the reduced frequency as well as the depth of such acid challenges. This could be achieved by (a) inhibitors of acid production, (b) simple avoidance, between main meals, of food or drinks containing fermentable sugars, (c) the consumption of items that contain alternative sweeteners that are only weakly metabolized, if at all, by oral bacteria, or (d) stimulation of

saliva flow after main meals. Some of these strategies will now be considered in more detail.

**Fluoride** The principal mode of action of fluoride is to increase the resistance of enamel to demineralization and to promote remineralization. Fluoride can also inhibit bacterial growth, but usually at concentrations much higher than those found in dental plaque (Hamilton and Bowden, 1988). The anti-caries properties of fluoride, therefore, have not generally been considered to involve its antimicrobial activity. At sub-MIC levels, however, fluoride can reduce glycolysis, while its antibacterial (Bradshaw et al., 1990) and anti-metabolic (Hamilton and Bowden, 1988) properties are enhanced markedly at low pH. Concentrations of fluoride that might be without effect at neutral pH could be inhibitory during acid production and thereby reduce the deleterious shifts in the plaque microflora by slowing the rate of change in pH. For this proposal to be tested, sub-MIC levels (1 mmol/L) of sodium fluoride were pulsed along with glucose into the nine-member mixed-culture system described earlier. This low level of fluoride prevented the selection of *S. mutans* under otherwise favorable growth conditions (Table 1). The cultures were still dominated by *L. casei* and *V. dispar* after 10 pulses, but the rate of acid production was reduced by fluoride, and acid-sensitive species persisted at higher levels than in the absence of fluoride (Bradshaw et al., 1990). Thus, fluoride was able to stabilize the composition of the microflora, at least partially, by reducing the pressure exerted by a rapid fall in environmental pH, confirming an earlier suggestion by Hamilton and Bowden (1982).

**Antimicrobial agents** Antimicrobial agents, such as chlorhexidine, can be used as an adjunct to mechanical cleaning for plaque control (Addy, 1986). Such agents are generally selected on the basis of their spectrum of inhibitory activity, and on their bactericidal or bacteriostatic mode of action (Lang and Brex, 1986). However, agents delivered from dental products have a relatively short half-life in the mouth and may be present for considerable periods at sub-MIC levels. At such concentrations, they might still serve a valuable function by interfering with bacterial metabolism, e.g., by inhibiting acid production (Scheie, 1989; Cummins, 1991; Marsh, 1992). This mode of action would be consistent with the ecological plaque hypothesis by again decreasing the impact of rapid changes in pH on both the stability of the microflora and on demineralization.

**Sugar substitutes and stimulation of saliva flow** Saliva is significant in a number of respects in terms of caries prevention. Its flow is important in the clearance of fermentable sugars from the mouth, while also providing buffering capacity to restore the pH of plaque to resting values. Furthermore, saliva contains antimicrobial factors, as well as urea and peptides from which base can be generated to raise the local pH; saliva can also remineralize enamel. Two approaches have been adopted to exploit these beneficial properties of saliva. First, the acid challenge to enamel, especially during between-meal periods, can be reduced by the consumption of snack foods, drinks, and confectionery that contain non-metabolizable sweeteners (sugar substitutes). The sweetness of these agents stimulates saliva flow in the absence of significant acid production, and this can promote remineralization of enamel. Some sugar substitutes—such as aspartame, saccharin, and xylitol—are also able to inhibit bacterial growth (Grenby and Saldanha, 1986), with

saccharin and xylitol being particularly effective against mutans streptococci (Best and Brown, 1987; Makinen, 1989; Scheie, 1989). Second, saliva flow can be stimulated for extended periods after a meal—for example, by the chewing of gum sweetened with sorbitol (Jensen, 1986; Jensen and Wefel, 1989). It has been proposed that the regular use of sorbitol chewing gum is not only non-cariogenic but also therapeutic, in that the stimulation of saliva flow can promote remineralization (Leach et al., 1989). Xylitol-containing chewing gum can be used in a similar manner; indeed, in general, the regular consumption of xylitol-containing gum gives a greater reduction in caries than similar sorbitol-containing products (Makinen, 1989). Thus, both approaches reduce the anti-homeostatic effect of low pH on the balance of the plaque microflora.

**Strategies to prevent periodontal diseases** Most conventional methods of treating disease involve mechanical removal of subgingival plaque and, sometimes, the use of antimicrobial agents, especially in advanced or refractory periodontal disease. Despite less being known about specific factors that result in changes in the subgingival microflora, an alternative (ecological) approach would be to alter the environment of the pocket to prevent the growth of the putative pathogens.

**Anti-inflammatory and antimicrobial agents** Anti-inflammatory agents might break the cycle of tissue destruction caused by both bacterial and host-derived proteases (Johnson and Curtis, 1994). This would also reduce the supply of GCF, and thereby restrict the availability of nutrients essential for the growth of some periodontopathogens. Some of the antimicrobial agents being used in dental

health products contain broad-spectrum antimicrobial agents which can reduce plaque, especially at sites that are difficult to clean. Care has to be taken with the regular, unsupervised use of such agents, so that the natural ecology of dental plaque will not be disrupted (Page, 1989). However, recent studies have suggested that, at their concentrations in the mouth, the activity of some of these agents may be more selective than hitherto suspected. For example, in the same way that chlorhexidine can be used for selective suppression of mutans streptococci in plaque (Kohler et al., 1984), so Triclosan and zinc citrate, in combination, have been found to have greatest activity against periodontopathogens *in vitro* (Bradshaw et al., 1993) and *in vivo* (Jones et al., 1990), while leaving streptococci associated with sound enamel and a healthy periodontium relatively unaffected (Bradshaw et al., 1993). Again, at sub-MIC levels, several antimicrobial agents may have additional potentially valuable properties by inhibiting bacterial proteases implicated in tissue destruction (Scheie, 1989; Cummins, 1991; Marsh, 1992).

**Oxygenating and redox agents** Another approach has been to try to raise the redox potential of the pocket (which is lowered during disease) to create an environment incompatible with the growth of obligate anaerobes. This has been tried with molecular oxygen or an oxygenating agent (Chasens, 1978), with various rates of success. Recently, the use of redox dyes has been proposed which, while not releasing oxygen, can raise the redox potential of an ecosystem (Wilson et al., 1992). Methylene blue was applied subgingivally on a daily basis for 7 days at 25 test sites; control sites in the same patients received water. Treatment led to a significant reduction in flow of GCF, and reduced the proportions of obligate anaerobes and motile organisms in the subgingival microflora; this was accompanied by a concomitant increase in facultatively anaerobic and coccal bacteria (Wilson et al., 1992). *In vitro* studies also demonstrated that methylene blue could raise the Eh of pre-reduced culture medium, and significantly reduce the viable counts of a

suspension of *P. gingivalis* (Fletcher and Wilson, 1993). These early studies confirm the theoretical basis of the ecological plaque hypothesis by showing that a preventive strategy that interferes with a critical event in the breakdown of microbial homeostasis in plaque can shift the ecological balance of plaque back toward that which is compatible with dental health.

**Strategies to enhance colonization resistance** The phenomenon by which one member of an ecosystem can inhibit the growth of another member is termed "bacterial interference". The possibility that antagonistic organisms could be used to control pathogens and prevent disease has been proposed for over 100 years and is termed "replacement therapy". This approach has the potential advantage that it provides life-long protection with minimal cost or compliance on behalf of the recipient, once colonization by the "effector" strain has been achieved (Hillman and Socransky, 1989). There are two main approaches by which replacement therapy is being considered as a means of enhancing colonization resistance in plaque to prevent caries and periodontal diseases.

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**Pre-emptive colonization** In this approach, ecological niches (functions) within plaque are filled by a harmless or potentially beneficial organism before the undesirable strain has had an opportunity to colonize or become established. The initial colonizer becomes integrated into the ecosystem and subsequently excludes the pathogen (Donoghue, 1990). Low-virulence mutants of mutans streptococci have been produced that are deficient in glucosyltransferase, intracellular polysaccharide, or lactate dehydrogenase (LDH) activity, and which are designed to prevent subsequent colonization by "natural" mutans streptococci. However, wild-type revertants can occur, the degree of colonization by the mutant can vary with the animal host, and mutants do not always compete successfully in vivo.

**Competitive displacement** An alternative approach has been to derive a more competitive strain that would displace a pre-existing organism from plaque (Donoghue, 1990).

Competitive displacement is of potentially greater clinical value, since it is not dependent on treatment with the "effector" strain at or before colonization by the undesired organism (Hillman and Socransky, 1989). An unusual strain of *S. salivarius* (TOVE-R) was shown to displace *S. mutans* from the teeth of rats and to inhibit tooth decay (Tanzer, 1989), but this organism was less effective when attempts were made to implant it into human plaque (Fisher and Tanzer, 1984). A strain of *S. mutans*, selected on the basis of enhanced bacteriocin production, was able to colonize the teeth of human volunteers persistently, and it reduced the indigenous strain of *S. mutans* in some subjects (for a review, see Hillman and Socransky, 1989). The properties of a number of potential "effector" strains for replacement therapy have been reviewed (Tanzer, 1989). Competitive displacement has also been considered in the treatment of LJP. As stated earlier, plaque from periodontally healthy sites contained organisms, such as H<sub>2</sub>O<sub>2</sub>-producing strains of *S. sanguis*, that inhibited the growth of *A. actinomycetemcomitans*, whereas the converse was true with plaque taken from sites with LJP (Hillman and Socransky, 1982). Levels of *A. actinomycetemcomitans* were reduced markedly in gnotobiotic rats when the animals were superinfected by wild-type *S. sanguis*, but variable findings have been reported when attempts have been made to implant *S. sanguis* in humans (Hillman and Socransky, 1989).

Thus, at present, conventional approaches using debridement and antibiotics remain the optimal form of treatment. Before replacement therapy can be considered as a practical alternative to existing treatment, the problems of implanting effective "effector" strains will have to be overcome, and assurances of the safety of these strains will be required. Molecular biology techniques are being exploited to develop suitable "effector" strains with the desired properties. Nevertheless, the use of bacterial interference to produce plaque either with a lower disease potential or with an increased level of colonization resistance would be consistent with the principles of the ecological plaque hypothesis.

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**23. Which statement is the MOST correct:**

- Efficient brushing and flossing always will prevent gingival inflammation.
- Periodontal disease is often the result of gut dysbiosis.
- Tooth decay is always the result of improper brushing and flossing.
- Bacteria in the gut are unique because they are unable to communicate with the microbiome in other parts of the body.

## **Activity of inflammatory bowel disease influences the expression of cytokines in gingival tissue.**

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### **Abstract**

This study assessed the cytokine expression in gingival and intestinal tissues from periodontitis patients with inflammatory bowel disease (IBD) and evaluated if IBD activity is a covariate to the amount of gingival cytokines. Paired gingival and intestinal tissues were collected from 21 patients and homogenised using a cell disruptor. Cytokine expression (IL-1 $\beta$ , IL-4, IL-6, IL-10, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IL-17A, IL-17F, IFN- $\gamma$ , sCD40L, and TNF- $\alpha$ ) was evaluated using bead-based multiplex technology. An inflammation score was developed using the intestinal cytokines that showed good accuracy to discriminate IBD active patients from those in remission and then a similar score was applied to gingival tissue. IL-4, IL-10 and IL-21 expressions were significantly increased in gingival tissue from patients with an active disease as compared to those with a disease in remission. The inflammation score (mean value of IL-1 $\beta$ , IL-6, IL-21, and sCD40L) was significantly higher in gingival tissue from patients with IBD activity. There was a significant correlation between gingival and intestinal inflammation scores ( $\rho=0.548$ ;  $P=0.01$ ). Significantly higher IL-23 and IFN- $\gamma$  levels and lower IL-31 and TNF- $\alpha$  levels were observed in gingival tissues than in intestinal ones. Activity of inflammatory bowel disease influenced the cytokine expression in gingival tissue.

**24. Which statement is incorrect:**

- a. LPS can pass from the gut into the circulatory system causing metabolic endotoxemia.
- b. The gut barrier can only be damaged by gluten.
- c. Metabolic endotoxemia can be reduced with spore-based probiotics.
- d. A “leaky periodontal pocket” must be treated concurrently with a “leaky gut”.

Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers

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Author contributions: McFarlin BK designed the study, collected data, interrupted findings, and prepared manuscript; Henning AL, Bowman EM, Gary MA and Carbajal KM collected data, interrupted findings, and prepared manuscript.

Institutional review board statement: The study was reviewed and approved by the UNT Institutional Review Board for Human Subjects Research.

Informed consent statement: Subjects provided written and oral consent to participate using an IRB-approved informed consent form specific to the study in question.

Conflict-of-interest statement: The present study was funded in part by a competitive research grant from Microbiome Labs, LLC (Glenview, IL) to the University of North Texas. The UNT team did not receive direct funding associated with the completion of the present study. The funding agency was not involved in the data collection, analysis, interpretation, and manuscript preparation. Double blind procedures and confidentiality were used to conduct the present study in a sound and unbiased manner. As such, the authors report no conflict of interest associated with completing the present study.

Data sharing statement: None.

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Abstract AIM To determine if 30-d of oral spore-based probiotic supplementation could reduce dietary endotoxemia.

**METHODS** Apparently healthy men and women (n = 75) were screened for post-prandial dietary endotoxemia. Subjects whose serum endotoxin concentration increased by at least 5-fold from pre-meal levels at 5-h post-prandial were considered “responders” and were randomized to receive either placebo (rice flour) or a commercial sporebased probiotic supplement [*Bacillus indicus* (HU36), *Bacillus subtilis* (HU58), *Bacillus coagulans*, and *Bacillus licheniformis*, and *Bacillus clausii*] for 30-d. The dietary endotoxemia test was repeated at the conclusion of the supplementation period. Dietary endotoxin (LAL) and triglycerides (enzymatic) were measured using

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Prospective Study

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an automated chemistry analyzer. Serum disease risk biomarkers were measured using bead-based multiplex assays (Luminex and Milliplex) as secondary, exploratory measures. **RESULTS** Data were statistically analyzed using repeated measures ANOVA and a  $P < 0.05$ . We found that spore-based probiotic supplementation was associated with a 42% reduction in endotoxin ( $12.9 \pm 3.5$  vs  $6.1 \pm 2.6$ ,  $P = 0.011$ ) and 24% reduction in triglyceride ( $212 \pm 28$  vs

$138 \pm 12$ ,  $P = 0.004$ ) in the post-prandial period. Placebo subjects presented with a 36% increase in endotoxin ( $10.3 \pm 3.4$  vs  $15.4 \pm 4.1$ ,  $P = 0.011$ ) and 5% decrease in triglycerides ( $191 \pm 24$  vs  $186 \pm 28$ ,  $P = 0.004$ ) over the same post-prandial period. We also found that sporebased probiotic supplementation was associated with significant post-prandial reductions in IL-12p70 ( $24.3 \pm 2.2$  vs  $21.5 \pm 1.7$ ,  $P = 0.017$ ) and IL-1 $\beta$  ( $1.9 \pm 0.2$  vs  $1.6 \pm 0.1$ ,  $P = 0.020$ ). Compared to placebo post supplementation, probiotic subject had less ghrelin ( $6.8 \pm 0.4$  vs  $8.3 \pm 1.1$ ,  $P = 0.017$ ) compared to placebo subjects.

**CONCLUSION** The key findings of the present study is that oral sporebased probiotic supplementation reduced symptoms indicative of “leaky gut syndrome”.

**Key words:** Metabolic endotoxemia; Chronic disease; Leaky gut syndrome; Probiotics; Multiplex; Cardiovascular disease; Inflammatory cytokines; High-fat meal challenge

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**Core tip:** Dietary or metabolic endotoxemia is a condition that affects approximately 1/3 of individuals living in Western society. It is characterized by increased serum endotoxin concentration during the first five hours of the post-prandial period following consumption of a meal with a high-fat, high-calorie content. The key findings of the present study, were that 30-d of oral spore-based probiotic supplementation reduced the incidence of dietary endotoxemia, which may be indicative of reduced gut permeability.

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**INTRODUCTION** Incidence of gastrointestinal (GI) distress and permeability has increased in prominence in modern society due in large part to the excessive consumption of

highly processed, calorie dense, commercially available foods[1]. These same dietary choices coupled with low physical activity are believed to be the primary causes underlying the current obesity epidemic[2]. Recent efforts have focused on the use of over-the-counter probiotics (typically *Lactobacillus* and *Bifidobacterium*) to address symptoms associated with GI abnormalities[3-5]. The lay literature has generally identified a goal of improved “GI health”, but unfortunately this is so broadly defined that it is nearly impossible to identify a single research focus[6]. Further complicating matters is that probiotic supplementation does not yield consistent results[7,8]. We have speculated that if an individual doesn’t have a pre-existing GI abnormality then they would not be a “responder” to probiotic supplementation. Complicating oral probiotic supplementation efforts is the fact that few traditional probiotic supplements (i.e., *Lactobacillus* and *Bifidobacterium*) deliver fully viable bacteria to the small intestine[9,10]. Recently it has been speculated gram positive, spore-forming probiotic strains may be a good alternative because the endospores that encapsulate the strains are highly resistant to stomach acid, potentially resulting in the delivery of more viable probiotics to the small intestine[11,12]. Thus, it appears that two major limitations of the existing probiotic literature lie with an inability to identify “responder” subjects prior to enrollment and issues associated with viable probiotic delivery to the small intestine. Dietary or metabolic endotoxemia occurs when one’s dietary consumption causes disruption in either GI permeability, the microbiota profile, or both[1,2,4,13-15]. Dietary endotoxemia transiently increases systemic inflammation, which chronically may increase one’s risk of a variety of diseases[2]. Our laboratory and others have demonstrated that consumption of a single, highfat, high-calorie meal was associated with an increase in serum endotoxin, triglycerides, metabolic biomarkers, inflammatory cytokines, endothelial microparticles, and monocyte adhesion molecules[16-22]. The post-prandial time course varies for each biomarker, but generally the transient changes occur during the first five hours of the post-prandial period. Given the direct link between nutrition, microbiota, GI permeability, and disease risk, our laboratory and others have speculated that these changes represent an appropriate treatment target for a probiotic intervention[23,24]. To address known issues with sufficient probiotic delivery, we utilized a “spore-based” probiotic in the present study. According to the literature the biggest advantages of a “spore-based” probiotic is that it is composed of endospores which are highly resistant to acidic pH, are stable at room temperature, and deliver a much greater quantity of high viability bacteria to the small intestine than traditional probiotic supplements[11,12]. To our knowledge, the present study is the first attempt to clinically leverage the benefits of spore-based probiotics to improve health outcomes. The primary purpose of the present study was to determine if 30-d of spore-based probiotic supplementation reduced post-prandial endotoxemia and triglycerides.

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The study enrollment was unique in that we developed an additional level of screening to only enroll subjects who had dietary endotoxemia (i.e., responders). Our secondary purpose was to determine if other metabolic biomarkers and cytokines, known to change after consuming a high-fat meal, would also be modified by 30-d of spore-based probiotic supplementation.

**MATERIALS AND METHODS** Determination of appropriate sample size All the procedures described in the present study were reviewed and approved by the University of North

Texas Institutional Review Board (IRB) for Human Subject's Research. Subjects provided their written and verbal consent to participate before being enrolled in the study. The present study was completed following a completion of a preliminary proof of concept study in the laboratory (data not shown). From this data, we identified that only 2 of 6 subjects ("responders") had a measurable dietary endotoxemia response (i.e., at least a 5-fold increase from pre-meal values at 5-h postprandial). "Responder" subjects experienced a 30% reduction in serum endotoxin (effect size = 0.40) at 5-h post-prandial following a 30-d probiotic intervention (same probiotic used in the present study). Based on these criteria, we identified that we needed to enroll a minimum of n = 10 "responders" in placebo and spore-based probiotic groups (n = 20 total) in order to achieve at least 80% statistical power. Eighty subjects were screened for a dietary endotoxin response, and 25 "responders" were enrolled (Table 1) and matriculated through the study treatments (Figure 1).

**Additional subject screening** Prior to testing for the post-prandial endotoxemia response, subjects also completed a series of other tests to exclude for other pre-existing conditions. Screening included measurement of body composition (whole body DEXA scan; GE Lunar Prodigy, United States), medical history assessment, and resting metabolic rate (RMR; MGC Diagnostics Ultima; St. Paul, MN, United States). Subjects who were currently taking or had taken in the previous 6-mo medications for the treatment of metabolic disease, antibiotics, probiotic supplements, anti-inflammatory medications, and/or daily consumed at least 3 serving of yogurt were excluded from further participation. Within the medical history, we also excluded subjects who were currently being treated for metabolic disease (i.e., diabetes mellitus), currently being treated for cardiovascular disease, and/or were obese (by BMI and/or percent body fat from DEXA). Individuals who met the initial screening criteria were scheduled to consume the experimental meal challenge on a separate day. The experimental meal challenge was used to identify subjects with a dietary endotoxin response that we considered "responders". Individuals classified as "responders" were enrolled in the supplementation phase of the study.

**Identification of "responders"** Experimental meal challenge: Subjects reported to the laboratory between 0600 and 1000 following an overnight fast (> 8-h) and abstention from exercise (> 24-h). Following collection of a pre-meal blood sample, subjects were provided a high-fat meal (85% of the daily fat RDA and 65% of the daily calorie needs based on RMR). Thin crust cheese pizza from a local vendor was used as the high-fat meal source (Table 2). Blood samples were measured for endotoxin concentration after the meal and only those subjects

Expressed interest ( n = 137)

Scheduled for laboratory screening ( n = 123)

Enrolled ( n = 80)

Randomized ( n = 32)

Placebo ( n = 13)

Probiotic ( n = 15)

Excluded, pre-screening ( n = 47)

Excluded, lab screening ( n = 43)

No dietary endotoxemia ( n = 48)

Non compliant ( n = 4)

Overall compliance 95%

Figure 1 Represents the consort diagram for the study that indicates the number of participants that matriculated through the study. Subjects were carefully screened for exclusion/inclusion criteria and if qualified were enrolled in the study. Consistent with our preliminary data 2 out of every 6 subjects presented a dietary/metabolic endotoxin response following consumption of the high-fat meal. A total of 26 individuals were identified to have the “responder” phenotype and were randomized to participate in either the probiotic or placebo condition.

Table 1 Subject characteristics

Characteristic Placebo ( n = 13)

Characteristic	Placebo ( n = 13)	Probiotic ( n = 15)
Age (yr)	21.8 ± 0.7	21.2 ± 0.5
Height (cm)	167.9 ± 3.2	170.8 ± 2.7
Body mass (kg)	74.2 ± 6.6	71.2 ± 3.1
Body mass index (kg/m <sup>2</sup> )	25.9 ± 1.5	24.3 ± 0.9
Body fat (%)	27.8 ± 4.1	25.2 ± 3.0
Fat mass (kg)	21.0 ± 4.3	17.3 ± 2.4
Lean mass (kg)	50.1 ± 3.8	50.0 ± 3.7
Bone mineral mass (kg)	2.9 ± 0.2	2.9 ± 0.1
Resting energy expenditure (kcal/d)	2243 ± 304	2071 ± 108

Values represent group mean ± SEM. No significant differences existed between groups with respect to subject characteristics.

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whose endotoxin level increased by > 5-fold at 5-h post-prandial were classified as “responders” and enrolled in the supplementation phase of the study. This same experimental meal challenge was completed at the end of the supplementation period to assess the effectiveness of spore-based probiotic supplementation at modifying the serum endotoxin response.

Supplementation conditions: “Responder” subjects were randomized to either a placebo (rice flour) or spore-based probiotic (Megasporebiotic; Physicians Exclusive, LLC; Glenview, IL, United States) condition. The spore-based probiotic included 4 billion spores from gram-positive, spore-forming strains [*Bacillus indicus* (HU36), *Bacillus subtilis* (HU58), *Bacillus coagulans*, and *Bacillus licheniformis*, *Bacillus clausii*]. Subjects were instructed to consume 2 capsules each day for a total of 30-d. Subjects were asked to promptly report any missed doses. Based on subject reporting, efficacy of intake was > 95% for the study period. All group assignments were completed using double-blind procedures. Subjects were instructed to maintain their habitual dietary and lifestyle habits during the study.

Blood sample collection: Venous blood samples were collected prior to the high-fat meal (PRE), 3-h, and 5-h post meal from a peripheral arm vein into an evacuated serum tube. Serum tubes were held at room temperature for 30-min to allow for clotting. Serum was separated by centrifugation and frozen at -80 °C until additional analysis.

Dietary endotoxin measurement Serum was analyzed for endotoxin concentration using a commercially available kinetic limulus amoebocyte lysate (LAL) assay (Lonza; Allendale, NJ, United States). Briefly, serum samples were diluted 1:100 in endotoxin-free water and heated at 70 °C for 15-min to remove contaminating proteases. Treated samples were then analyzed in triplicate using an automated chemistry analyzer (Chem Well T; Palm City, FL, United States) to determine endotoxin concentration against an *E. coli* endotoxin standard.

Serum triglyceride measurement Serum was analyzed in triplicate for triglyceride concentration using an endpoint enzymatic assay (Pointe Scientific; Canton, MI, United States) on an automated chemistry analyzer (ChemWell T).

Exploratory disease risk biomarkers: Previously frozen serum samples were analyzed as previously described[25-27]. Briefly, ghrelin, insulin, leptin, MCP-1, GMCSF, interleukin (IL)-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL12(p70), IL-13, and tumor necrosis factor alpha (TNF- $\alpha$ ) were measured in duplicate using a commercially available bead-based multiplex assay (Milliplex; MilliporeSigma; St. Louis, MO, United States) and an automated analyzer (Luminex MagPix; Austin, TX, United States). Raw data files were used to calculate unknowns from standards using Milliplex Analyst software (MilliporeSigma).

Statistical analysis Prior to formal statistical testing data were assessed for normality.

Non-normal data was log-transformed to stabilize this assumption prior to formal testing.

Data were analyzed using a condition (placebo or probiotic)  $\times$  experiment time (baseline and 30-d post)  $\times$  meal time (pre, 3, and 5-h post) analysis of variance (ANOVA) with repeated measurements on the 2nd and 3rd factors. P-values were adjusted using the Huynh-Feldt method to account for the repeated measures design. Significance was set at  $P < 0.05$ . Location of significant effects was determined using separate t-tests with a Bonferroni correction for multiple comparisons. In order to visualize the responses collectively, we log transformed all the responses to normalize the various biomarkers to a similar scale. We then created three radar plots (one for each sampling time point). Each plot contained the log transformed variable response at baseline and 30-d post and a third line for the fold-change from pre-meal response). Heat maps were generated for variables that showed similarity to endotoxin responses using a three-color approach: Red (large increase from pre-meal), yellow (intermediate response), and green (large decrease from pre-meal) (Figure 2). We have used a similar approach to data visualization in past manuscripts and this is an effective and accepted method[19,28].

**RESULTS** Endotoxin and triglycerides We found significant three-way interaction effects for both serum endotoxin ( $P = 0.011$ ; Figure 3A) and triglycerides ( $P = 0.004$ ; Figure 3B). In each instance, there was no difference between the post-prandial response between the two treatment groups (i.e.,

Table 2 Meal composition

Component Placebo ( n = 13)

Probiotic ( n = 15)	Total calories (kcal)	1630.4 $\pm$ 134.4	1644.7 $\pm$ 94.5	Total caloric needs
	(% of RMR)	72%	79%	Servings (#)
		6.3 $\pm$ 0.5	6.4 $\pm$ 0.4	Fat (g)
		88.8 $\pm$ 7.3	89.6 $\pm$ 5.1	Fat (kcal)
		799.3 $\pm$ 6.6	806.4 $\pm$ 46.3	Saturated fat (g)
		31.7 $\pm$ 2.6	32.0 $\pm$ 1.8	Trans fat (g)
		0	0	Protein (g)
		69.8 $\pm$ 5.8	70.4 $\pm$ 4.0	Carbohydrate (g)
		145.9 $\pm$ 12.0	147.2 $\pm$ 8.5	Carbohydrate (kcal)
		583.6 $\pm$ 48.1	588.8 $\pm$ 33.8	Cholesterol (mg)
		152.3 $\pm$ 12.5	153.6 $\pm$ 8.8	Sodium (mg)
		2911.9 $\pm$ 240.0	2937.4 $\pm$ 168.8	

Values represent group mean  $\pm$  SEM. No significant differences existed between groups with respect to meal composition.

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Pre	5-h post	Pre	5-h post	
20	18	16	14	12 10 8 6 4 2 0
Serum endotoxin (U/L)				
Spore-based probiotic Placebo				



associated with a 42% reduction in serum endotoxin at 5-h post-prandial compared to a 36% increase in placebo at the same time point. Sporebased probiotic supplementation was associated with a 24% reduction in serum triglycerides at 3-h postprandial compared to a 5% reduction in placebo at the same time point.

**Exploratory biomarkers** We found significant trial  $\times$  condition interactions for IL-12p70 ( $P = 0.017$ ; Figure 4A), IL-1 $\beta$  ( $P = 0.020$ ; Figure 4B), and ghrelin ( $P = 0.017$ ; Figure 4C). We also found potentially interesting trends for IL-6 ( $P = 0.154$ ; Figure 5A), IL-8 ( $P = 0.284$ ; Figure 5B), and MCP-1 ( $P = 0.141$ ; Figure 5C). These effects were consistent with the pattern observed for serum endotoxin in that sporebased probiotic intervention was associated with a reduction in a given biomarker at post-supplementation compared to pre-supplementation and placebo.

**DISCUSSION** An a priori review of the existing literature[5,29], lead our team to speculate that there may be an ideal subject phenotype that was “responsive” to sporebased probiotic treatment. Thus, we designed and implemented a screening protocol for the present study to identify individuals who presented with postprandial endotoxemia at baseline, which may be a hallmark sign of intestinal permeability and “leaky gut” syndrome[14,15,22,23]. We believe our approach to subject selection increased the efficacy and applicability of our key findings. Within our “responder” population (who likely had a non-protective microbiome), we were able to demonstrate that 30-d of oral supplementation with a viable, spore-based probiotic was associated with a significant reduction in post-prandial endotoxin and triglycerides. Further, we found that several of our exploratory biomarkers were either significantly reduced (IL-12p70, IL-1 $\beta$ , and ghrelin) or trended toward reduction (IL-6, IL-8, and MCP-1) with sporebased probiotic supplementation. It is reasonable to speculate that the spore-based probiotic supplement may have exerted its effect by altering the gut microbial profile, altering intestinal permeability, or a combination of the two effects. The present study was designed to assess systemic changes rather than focus on intestinal measures that are invasive or impossible to make accurately in human subjects. The reductions observed

Pre 3-h post 5-h post Pre 3-h post 5-h post

35

30

25

20

15

10

5

0

IL-12-p70 (pg/mL)

Pre 3-h post 5-h post Pre 3-h post 5-h post

2.5

2.0

1.5

1.0

0.5

0.0

IL-1 $\beta$  (pg/mL)

Pre 3-h post 5-h post Pre 3-h post 5-h post  
 12  
 10  
 8  
 6  
 4  
 2  
 0

Ghrelin (pg/mL)

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Figure 4 Serum IL-12p70 (A), IL-1 $\beta$  (B), and ghrelin (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects consumed an oral probiotic supplement for 30-d and the experimental meal challenge was completed at baseline and following the 30-d supplementation period. Probiotic responses were compare to placebo. IL: Interleukin.

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in the present study with spore-based probiotic supplementation were consistent with a transient reduction in chronic disease risk. It is also important to note that the reported changes were observed while the collegeaged subjects continued to lead their habitual life with no directed modification. They continued to be exposed to many of the stressors that are known to negatively affect gut permeability in college-aged individuals (i.e., consumption of microwaved and other processed food, fast foods, soft drinks with their excess of sugars, including artificial sugars, colorings and flavorings, energy drinks, alcohol consumption, lack of sleep, exam anxiety, etc.). Previous authors consistently speculate that the onset and progression of chronic disease results from the accumulation of transient changes in ones' health that result from lifestyle choices[16,18,19,22,28,30-32].

Unfortunately, the current literature has yet to define the quantity of transient change that must be accumulated to cause disease onset. Instead, previous studies have attempted to use lifestyle modifications (i.e., nutrition, physical activity, etc.) to minimize negative changes in health. One such problem, especially in western cultures, is the wide accessibility to high-fat, high-calorie meals, creating an environment where excessive, low-quality nutritional

habits are the norm. In these diets elevated post-prandial endotoxin and triglyceride are consistently reported as problematic changes. Our observed baseline responses mirror previous reports[22,31-33]. Recently a review article touted the potential of probiotic supplementation to prevent metabolic or dietary endotoxemia[24], but to our knowledge no published study has yet to demonstrate this outcome. Thus, our finding of a 42% reduction in metabolic endotoxemia is novel and unique. Further interpretation of our finding does reveal a potentially interesting effect, while 30-d of supplementation reduced

metabolic endotoxemia by 42%, it did not completely prevent metabolic endotoxemia. It is plausible to speculate that a longer period of supplementation may result in greater reductions in metabolic endotoxemia. Cani et al [14,15] previously reported in rodents, that the only viable method to “reprogram” the gut microbial response was to initially treat animals with a broad-spectrum antibiotic. For obvious ethical reasons treating human subjects with antibiotics is likely not a viable experimental design consideration, but perhaps the same effect could be achieved with a longer period of probiotic supplementation. In addition to probiotic effects, we also observed an interesting response in placebo subjects. Specifically, the placebo subjects presented

Pre 3-h post 5-h post Pre 3-h post 5-h post

16

14

12

10

8

6

4

2

0

IL-6 (pg/mL)

Pre 3-h post 5-h post Pre 3-h post 5-h post

10 9 8 7 6 5 4 3 2 1 0 IL-8 (pg/mL)

Pre 3-h post 5-h post Pre 3-h post 5-h post

250

200

150

100

50

0

Ghrelin (pg/mL)

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Figure 5 Serum IL-6 (A), IL-8 (B), and MCP-1 (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects consumed an oral probiotic supplement for 30-d and the experimental meal challenge was completed at baseline and following the 30-d supplementation period. Probiotic responses were compared to placebo. While effects did not reach statistical significance, trends are consistent with other variables that did significant change (Figures 2 and 3). IL: Interleukin.

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with an even greater metabolic endotoxemia response following a 30-d period. We do not believe that this observation is due to the experimental treatment, but is rather likely due to a diurnal fluctuation in metabolic endotoxemia responses. Thus, placebo subjects trended toward increased metabolic endotoxemia, while probiotic intervention reversed that effect. Since the present 30-d probiotic intervention did not completely prevent metabolic endotoxemia, it is reasonable to speculate that an intervention longer than 30-d may be necessary to completely prevent metabolic endotoxemia. We have previously demonstrated that the consumption of a high-fat meal causes transient biological changes that were consistent with a transient increase in risk of atherosclerosis[16,18,21,32]. These changes combined with a post-prandial increase in serum triglycerides creates a milieu that favors foam cell formation and the development of atherosclerotic plaques[19,31,33,34]. In the present study, the baseline post-prandial meal response presented outcomes that were consistent with published data from our laboratory and others[16,18,19,22,31-33,35]. Thus, the present study presented an opportunity to assess if a probiotic intervention would change disease risk biomarkers in a similar manner as endotoxin and triglycerides. We found significance across the entire meal combined between conditions, but were unable to tease apart specific time point differences. A post hoc sample size analysis revealed that we would have needed to enroll approximately 20 “responder” subjects in each group to delineate specific time point changes for biomarkers. Regardless, we found significant reductions in IL-12p70, IL-1 $\beta$ , and ghrelin. Previous research has indicated that obese subjects do not have as great of a post-prandial suppression ghrelin than normal weight subjects[36]. The authors do not explain the nature of the change, but given the observations of the present study, it is reasonable to speculate that obesity status may very well effect the gut microbiome[36]. It is plausible that in the present study, without changing body weight, we were able to create the microbiome of a normal weight individual thus restoring normal post-prandial ghrelin responses. Given the pro-inflammatory actions of IL-1 $\beta$ , the observed reduction with probiotic supplementation was consistent with reductions in post-prandial systemic inflammation. Reduced ghrelin may be indicative of better post-prandial hunger/satiety control with probiotic. IL-12p70 has a variety of metabolic actions, the chief action in the present study is the ability to modulate the release of TNF- $\alpha$  or related inflammatory cytokines following antigenic challenge[37,38]. In the case of the present study, reduced IL-12p70 with probiotic supplementation may reflect a reduction in systemic inflammatory capacity. In addition to the biomarkers that reached significance, we also found similar numerical trends for IL-6, IL-8, and MCP-1, which are all released by adipose tissues and commonly elevated in obese individuals[27,31,39]. The biomarkers observed to change in the present study following the probiotic intervention are involved in the accumulation of systemic inflammation[38,40-43]. The existing literature has linked elevated systemic inflammation to the pathophysiology of cardiovascular and metabolic diseases, thus even a transient reduction in systemic inflammation biomarkers may be associated with reduced disease risk[2,24]. The biomarkers measured in the present study are most often measured in the context of long-term weight loss (> 12 wk) interventions. In those weight loss models, it can take up to 16-wk to reduce body weight enough that biomarkers change. It is interesting that we demonstrated similar reductions in inflammatory biomarkers in 1/4 the time, but also in the absence of weight loss. We have presented novel results

concerning the ability of probiotic supplementation to elicit transient effects. In summary, the key findings of the present study demonstrate that 30-d of spore-based probiotic supplementation resulted in a blunting of dietary endotoxin, triglycerides, and potentially systemic inflammation. To our knowledge, the present study is the first to report that a short-term spore-based probiotic intervention altered dietary endotoxemia in human subjects, although the effect has been widely reported in mice[1,14]. Due to limitations associated with using human subjects, it was not possible to directly measure gut permeability in the present study. Despite this, it is reasonable to speculate that the underlying cause of the observed reductions in post-prandial endotoxemia may be due to changes in the gut microbiome, gut permeability, or a combination of the two. Future research is needed to determine if a longer course of treatment with a spore-based probiotic results in additional health improvements.

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**COMMENTS** Background Dietary or metabolic endotoxemia is a condition that affects approximately 1/3 of individuals living in Western society. It is characterized by increased serum endotoxin concentration during the first five hours of the post-prandial period following consumption of a meal with a high-fat, high-calorie content. Long-term repeated dietary endotoxemia may increase the risk of developing a variety of chronic diseases via an inflammatory etiology. Of the available treatments, oral probiotic supplementation has been purported to reduce gastrointestinal (GI) permeability to endotoxin, which in theory should suppress the dietary endotoxin **COMMENTS**

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response.

Research frontiers GI health is a hot topic and there is great interest in the study of natural substances that have the potential to improve GI health. Probiotics have been studied with inconsistent results, the present study was designed to specifically address previous limitations

Innovations and breakthroughs For the purposes of this study the authors validated a new method for identifying subjects that may be “responders” to probiotic intervention. This screening method included only enrolling subjects that had at least a 5-fold increase in serum endotoxin at 5-h post prandial. Using this method of subject screening, the authors believe that the present study is the first published account demonstrating a significant reduction in post-prandial endotoxemia. It is also significant that the authors also found reductions in disease risk biomarkers after only 30-d of probiotic supplementation. The approach to subject screening for probiotic studies is a novel approach that the authors hope will become a standard for future studies in the area.

Applications To the knowledge the present study is the first published report that has conclusively documented that short-term probiotic supplementation can reduce the incidence of leaky gut syndrome. The authors believe that the lessons learned from this

study that will be critical to future projects is that: (1) detailed screening is needed to qualify subjects who are certain to respond; and (2) the type of probiotic used should be carefully selected. The present study used a spore-based probiotic that is known to have greater than 90% survivability after exposure to stomach acid. Survivability after exposure to stomach acid is a critical factor in the assessment of commercial probiotics that is often overlooked in the selection and study design.

Terminology Dietary or metabolic endotoxemia is defined as a rise in blood serum endotoxin concentration during the first five hours after eating a meal. This post-meal period is also known as the post-prandial period. The most common cause of dietary endotoxemia is a disruption of gut barrier function. There is currently no accepted clinical test in humans to measure for gut barrier function. Disrupted gut barrier function cannot be predicted by any combination of baseline measures. Thus, to the knowledge the only means by which to assess gut barrier function was to complete a dietary endotoxemia test used in the present study. The endotoxin measured in the blood comes from bacteria that populate the GI track.

#### Regulation of Gut Microbiota and Metabolic Endotoxemia with Dietary Factors

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**Abstract:** Metabolic endotoxemia is a condition in which blood lipopolysaccharide (LPS) levels are elevated, regardless of the presence of obvious infection. It has been suggested to lead to chronic inflammation-related diseases such as obesity, type 2 diabetes mellitus, non-alcoholic fatty liver disease (NAFLD), pancreatitis, amyotrophic lateral sclerosis, and Alzheimer's disease. In addition, it has attracted attention as a target for the prevention and treatment of these chronic diseases. As metabolic endotoxemia was first reported in mice that were fed a high-fat diet, research regarding its relationship with diets has been actively conducted in humans and animals. In this review, we summarize the relationship between fat intake and induction of metabolic endotoxemia, focusing on gut dysbiosis and the influx, kinetics, and metabolism of LPS. We also summarize the recent findings about dietary factors that attenuate metabolic endotoxemia, focusing on the regulation of gut microbiota. We hope that in the future, control of metabolic endotoxemia using dietary factors will help maintain human health.

**Keywords:** metabolic endotoxemia; lipopolysaccharide; gut microbiota; dietary factors

1. Introduction Lipopolysaccharide(LPS)isacomponentoftheoutermembraneofgram-negativebacteriaandis known to induce a variety of inflammatory reactions through Toll-

like receptor 4 (TLR4). Injection of LPS into human blood elicits an inflammatory response [1,2], but it was thought that LPS is rarely detected in human blood, except under pathological conditions such as infection and colitis. However, in 2007, Cani et al. showed that mice fed with a high-fat diet had higher blood LPS levels than normal chow-fed mice, resulting in inflammation of the liver and adipose tissue, which led to the development of NAFLD and insulin resistance, and the authors defined this condition as metabolic endotoxemia [3].

Since then, studies on metabolic endotoxemia have been conducted for a variety of diseases. It has been reported that blood LPS levels are higher in humans with obesity [4], type 2 diabetes [5], NAFLD [6], pancreatitis [7], amyotrophic lateral sclerosis [8], and Alzheimer's disease [8] than those in healthy individuals. Although the causal relationship between metabolic endotoxemia and disease onset is unclear, it is expected to be an interesting target in the future from the viewpoint of disease prevention and treatment. In recent years, the association between metabolic endotoxemia and dietary factors, and the mechanism by which fat intake induces metabolic endotoxemia have been actively studied. In contrast, dietary factors that suppress metabolic endotoxemia have also been explored. Here, we review the relationship between fat intake and induction of metabolic endotoxemia, focusing on gut dysbiosis

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and the influx, kinetics, and metabolism of LPS. We also summarize the recent findings in humans and animals about dietary factors that attenuate metabolic endotoxemia, focusing on regulation of gut microbiota.

## 2. Fat Intake and Metabolic Endotoxemia

### 2.1. Dysbiosis

As Cani et al. reported an increase in blood LPS levels due to a high-fat diet in mice, the mechanism of LPS influx by fat ingestion has been investigated. LPS content both in cecal contents and blood was concomitantly increased by fat ingestion [9], and this increase of LPS was suppressed with oral administration of intestinal alkaline phosphatase, a LPS inactivating enzyme [9]. Oral administration of ampicillin and neomycin, broad-spectrum antibiotics that are poorly absorbed, also suppressed the increase in blood LPS concentration induced by a high-fat diet [10].

These reports suggest that intestinal bacteria are an important source of LPS. In particular, Cani et al. demonstrated changes in intestinal flora (reduction in *Bacteroides*, *Bifidobacterium*, and *Eubacterium*) due to a high-fat diet. Thus, dysbiosis of the intestinal flora due to a high-fat diet has attracted attention as a possible cause for metabolic endotoxemia. Changes in the intestinal bacteria due to ingestion of a high-fat diet have been studied in animals and humans and have been summarized in a review by Netto Candido et al. [11]. In animals, it has been reported that a high-fat diet increases the proportion of Firmicutes, Proteobacteria, and the ratio of Firmicutes to Bacteroidetes. On the other hand, in humans, it has been reported that high-fat dietary intake increases the proportion of Bacteroidetes and decreases the proportion of Firmicutes and Proteobacteria. One possible cause of the different changes in the gut microbiota at the phylum level (e.g., Firmicutes, Bacteroidetes, Proteobacteria) in human and animal studies is the difference in the type of fat consumed. The high-fat diet used in animal experiments (e.g., Research Diets Inc., catalog# D12451) contains lard, while human studies assess fat intake in daily diets. Devkota et al. evaluated the gut microbiota in C57BL/6 mice fed a low-

fat diet, a high-fat diet with lard, or a high-fat diet with milk fat for 21 days [12]. In this experiment, both high-fat diets were isocaloric, rich in saturated fatty acids, and 37% of the ingested kcal were from fat. As a result, the proportion of Firmicutes increased and that of Bacteroidetes decreased in the gut microbiota of mice fed a high-fat diet containing lard, compared to mice fed a low-fat diet. In contrast, in mice fed a high-fat diet containing milk fat, the proportion of Firmicutes decreased and that of Bacteroidetes increased compared to the low-fat diet fed mice. Interestingly, Devkota et al. also identified specific bacteria that increased only by ingestion of a high-fat diet containing milk fat [12]. Compared to mice fed a low-fat diet, or a high-fat diet containing lard, mice fed with a high-fat diet containing milk fat had increased proportions of *Bilophila wadsworthia*, a sulfite-reducing bacterium, in gut microbiota. They also elucidated the mechanism underlying this increase; intake of milk fat increased the level of taurocholic acid in bile. *Bilophila wadsworthia* populations increased by utilizing sulfur components in taurocholic acid, causing intestinal inflammation in mice. An increase in total fecal bile acid and a concomitant increase in *Bilophila wadsworthia* in the gut microbiota was also reported in humans upon dietary intake of animal fat [13]. Natividad et al.

also showed that increased *Bilophila wadsworthia* in mice fed a high-fat diet contributed to increased blood LPS levels (they measured soluble CD14 as a surrogate marker), increased fasting blood glucose levels, and the development of a fatty liver [14]. As *Helicobacter pylori* was discovered as a pathogen in gastric cancer, some pathobionts may also exist for induction of metabolic endotoxemia (however, this cannot be detected by evaluating changes of the gut flora at the phylum levels). We further discuss the bacterial genera that are thought to be associated with metabolic endotoxemia in Section 4. It is also necessary to consider dietary LPS as a source of LPS. For example, milk has been reported to contain high concentrations of LPS in some commercial products [15]. Multiple animal studies have reported that ingested LPS may contribute to increased blood LPS levels. Specifically, Kaliannan et al. measured blood LPS levels 45 min after ingestion of LPS alone or corn oil and LPS in mice [9]. It showed that blood LPS levels were elevated when corn oil and LPS were co-administered.

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Lindenberg et al. reported that LPS concentrations in the blood were higher in mice fed a high-fat diet containing LPS than in mice fed a high-fat diet without LPS [16]. However, the effect of LPS levels in food on blood LPS levels has not been adequately studied in humans and further studies are needed.

**2.2. Mechanisms of the Influx of LPS into the Bloodstream** The gut is protected by a barrier consisting of a mucin layer and epithelial cells. Thus, even if the number of gram-negative bacteria that produce LPS increases in the gut, it is unlikely that the bacterium itself will invade the body. The limulus amoebocyte lysate assay used to measure LPS recognizes lipid A, a glycolipid moiety of LPS [17], but because lipid A is embedded in the outer membrane of gram-negative bacteria [18], elevated blood LPS levels suggest that LPS released from gram-negative bacteria is flowing into the blood. In an *in vitro* study with *Escherichia coli*, the concentration of free LPS in the culture medium increased with bacterial growth, but the addition of antibiotics stimulated further LPS release [19]. In addition, Jin et al. suggested that treatment with penicillin and erythromycin killed the gram-negative bacteria, *Bacteroides* and  $\gamma$ -Proteobacteria, leading to increased blood LPS levels in mice [20]. Radilla-Vázquez et al.

conducted a correlation analysis of blood LPS levels with fecal *Escherichia coli*, *Prevotella*, and *Bacteroides fragilis* counts in humans and reported that the lower the number of gram-negative bacteria *Escherichia coli*, the higher the risk of increased blood LPS levels [21]. These reports suggest that LPS release by lysis as well as the increase in gram-negative bacteria may be important factors in increasing blood LPS levels, which may contribute to the inconsistent relationship between changes in intestinal flora and blood LPS levels described above. With respect to the influx of free LPS, Laugerette et al. reported that in an in vitro assay system using the intestinal epithelial cell line Caco-2, LPS permeability to the basal side was increased in the presence of oleic acid, 2-oleoylglycerol, soybean lecithin, cholesterol, and sodium taurocholate [22]. In addition, Clement-Postigo et al. reported a positive correlation between increased LPS levels in the chylomicron fraction and increased triglyceride concentration in serum up to 3 h after a high-fat meal [23]. LPS uptake in chylomicrons has been observed by immunoelectron microscopy [22]. These results suggest that released-LPS in the intestine is taken up into micelles during lipid absorption, and then LPS is absorbed from the intestine together with lipids. In mice, ingestion of a high-fat diet has been reported to increase intestinal permeability by inhibiting the mRNA expression of tight junction-related factors, zonula occludens-1 (ZO-1) and occludin in intestinal epithelial cells [10]. This increase in intestinal permeability is markedly inhibited by antibiotic administration [10], suggesting that it is not the direct effect of lipids but rather a change in intestinal flora. Indeed, secondary bile acids metabolized by enteric bacteria are known to inhibit expression of intestinal tight junction proteins [24,25]. Increased intestinal LPS has been reported to destroy the tight junction of intestinal epithelial cells through TLR4 [26]. Although ingestion of a high-fat diet broadly enhances intestinal and colonic permeability [27], permeability in the colon is closely related to increased blood LPS levels [28,29].

Therefore, disruption of the barrier function by a high-fat diet may have also contributed to the LPS inflow, and the colon may be important as a site of the absorption. The transit time of colonic contents is also probably important. In mice, Anitha et al. suggested that saturated fatty acids induced apoptosis of neurons in the large intestine, reduced peristalsis, induced constipation, and increased blood LPS levels [30]. On the other hand, Reichardt et al. similarly evaluated peristalsis of the large intestine by ingestion of a high-fat diet, but did not observe a clear decrease in peristalsis and an increase in blood LPS levels [31]. Anitha et al. and Reichardt et al. used high-fat diets where either 60% or 30%, respectively of ingested kcal came from fat. Although the ratio of fat to energy intake varied, it has been reported that blood LPS levels increased by consumption of a high-fat diet with 30% of kcal ingested being from fat [32,33]. Therefore, the reason for the lack of increase in blood LPS levels in the study of Reichardt et al. is not considered to be a difference in the fat content of the diet. Ingestion of a high-fat diet does not simply increase blood LPS levels, and retention time of colonic contents due to constipation may also contribute to absorption of LPS.

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**2.3. Kinetics and Activity of LPS** The LPS concentration in the portal blood is approximately 10 times higher than the LPS concentration in the peripheral blood [34], suggesting that a part of the LPS released in the intestinal tract is flowing from the portal vein. On the other hand, LPS which is concomitantly absorbed with lipids binds to lipoproteins in chylomicrons via LPS-binding protein (LBP) [35], and is thought to pass through the

lymphatic system, flow into the blood stream from the left subclavian vein, and then circulate throughout the body. It is reported that blood LPS is bound to various lipoproteins, with plasma LPS concentrations of 31%, 30%, 29%, and 10% for the very low-density lipoprotein (VLDL) fraction, low-density lipoprotein (LDL) fraction, high-density lipoprotein (HDL) fraction, and free LPS, respectively [36]. In addition, LPS bound to lipoproteins of HDL has been reported to be transferred to VLDL and LDL by LBP and phospholipid transfer protein [37], suggesting that the LPS concentration of each lipoprotein fraction changes actively. There are several reports that bioactivity of LPS bound to lipoprotein varies with the type of lipoprotein. First, Vreugdenhil et al. evaluated the effect of chylomicrons, HDL, LDL, and VLDL on the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) from human peripheral blood mononuclear cells on LPS stimulation and showed that chylomicrons inhibited TNF- $\alpha$  production the most [35]. Emansipator et al. reported that a mix of LPS with LDL or HDL decreased the spike recovery of LPS activity in the limulus amoebocyte lysate test, and that incubation of LPS with apo A1 decreased the febrile response of rabbits when injected compared to those without apo A1 [38].

In a study using human mononuclear cells [39] and the mouse macrophage cell line Raw 264.7 [40], it was reported that LPS bound to HDL showed reduced interleukin-6 (IL-6) and TNF- $\alpha$  production. VLDL has also been reported to inhibit LPS-induced activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) [41]. On the other hand, oxidized LDL has been shown to promote NF- $\kappa$ B activation with LPS in macrophages [42], suggesting that binding to lipoproteins not only decreases LPS activity but also may promote inflammatory responses. Increased LPS content has been reported in the livers of mice fed a high-fat diet [43], suggesting that the liver is an important site for LPS clearance. Ninety percent of the free LPS that entered the bloodstream is captured by liver resident macrophages (i.e., Kupffer cells) within 1 h [44]. LPS bound to HDL attaches primarily to sinusoidal epithelial cells of the liver [40,44], but it shows slower blood kinetics than free LPS, with 50% present in plasma even 1 h after administration and the amount accumulated in the liver accounted for only 15% of the dose [44]. LPS bound to HDL on the other hand is distributed widely to organs other than the liver, such as the kidney and adipose tissue [44]. LPS accumulated in the liver is inactivated by acyl hydroxylase produced by Kupffer cells regardless of free or HDL-bound form [44]. Previously, in a mouse model of high-fat diet plus streptozotocin-induced non-alcoholic steatohepatitis-hepatocellular carcinoma, fecal LPS levels were continuously elevated from six weeks, while liver LPS levels were transiently elevated at eight weeks, followed by increased plasma LPS levels [45].

This report suggests that the liver acts as the first barrier against LPS entering from the intestinal tract and that liver dysfunction leads to elevated blood LPS levels. Interestingly, LPS administration in mice increased the expression of apolipoprotein AIV in the liver via TLR4, suggesting that the liver has a mechanism to increase HDL production and protect itself against LPS stimulation [46].

### 3. Dietary Factors that Decrease Blood LPS Levels

Previous reports investigating the effects of dietary factors on blood LPS levels are summarized in Table 1 (human interventional studies), Table 2 (human epidemiological studies) and Table 3 (animal studies). The findings about representative food categories are reviewed in the following sections.

3.1. Probiotics Probiotics were defined by Fuller in 1989 as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” [47], and has been studied mainly for lactic acid bacteria and bifidobacteria. Since metabolic endotoxemia has been implicated in gut dysbiosis, the effects of probiotics have been investigated. However, the results in humans are unfavorable (Table 1). Lever et al. administered 195 mL of Yakult light (containing  $2 \times 10^{10}$  colony-forming unit (CFU) of *Lactobacillus casei* Shirota) for three months to individuals with metabolic syndrome. The absence of detectable blood LPS in this study led to an assessment of the surrogate LBP level, which was significantly higher in the Yakult light-fed group than in the non-fed group [48]. Pei et al. conducted a nine-week study in which low-fat yogurt was ingested in healthy or obese individuals, however, no significant decrease in blood LPS or LBP levels was observed [49]. In addition, Pei et al. studied whether low-fat yogurt could be administered before a meal to suppress the increase in blood LPS after a meal [50] and found no efficacy. On the other hand, there have been several reports of the efficacy of probiotics in animal studies (Table 3) [43, 51–55]. *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bacillus cereus* are used as species, and the dosage ranges from 10<sup>7</sup> to 10<sup>10</sup> CFU/day for four to twelve weeks. These animal studies used a high-fat diet, a high-fat high-sucrose diet, or a Zucker-Lepfa/fa obesity model. In addition to a significant decrease in blood LPS or LBP levels, improvement of obesity, glucose metabolism, and dyslipidemia was also observed. Since the effects of probiotics are strain-specific, it is expected that the effects of strains that have been effective in animal studies will be verified in humans.

3.2. Prebiotics Prebiotics was defined by Gibson and Roberfroid in 1995 as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health” [56], and among the food components, dietary fiber and oligosaccharides are known as typical prebiotics. To date, human intervention studies have been conducted with oligofructose [57], inulin [58, 59], galacto-oligosaccharides [60–62], resistant dextrin [63], insoluble dietary fiber [64], and whole grains (Table 1) [65]. Oligofructose is an oligosaccharide containing one molecule of glucose and several molecules of fructose and is found in many fruits and vegetables. Inulin is a type of fructose-polymerized polysaccharide that is abundant in vegetables such as burdock and onion. In intervention studies with oligofructose [57] and inulin [58, 59], subjects with obesity, overweight subjects, and subjects with type 2 diabetes consumed 10–21 g of test substances for 8–12 weeks. Two of the three studies showed a significant decrease in blood LPS levels [57, 58]. One study also showed a decrease in plasminogen activator inhibitor-1 (PAI-1), a risk indicator for thrombosis [57], and the other study showed an improvement in glucose metabolism [58]. Galacto-oligosaccharides are oligosaccharides in which multiple molecules of galactose are attached to one molecule of glucose. Similar to oligofructose, there have been three reports of interventional trials for galacto-oligosaccharide in obese, overweight, and type 2 diabetic patients. One study showed that galacto-oligosaccharides reduced blood LPS levels, and improved obesity by suppressing appetite [62]. In mice, chronic administration of LPS has been

reported to induce hyperphagia by decreasing leptin sensitivity of afferent vagal nerves [66], and the reduced blood LPS levels and appetite suppression seen with galacto-oligosaccharide administration are of interest in supporting an association between LPS and appetite.

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**3.3. Polyphenols** Polyphenols are secondary metabolites found in plants and are responsible for protection against oxidative stress, UV damage, and pathogenic microorganisms [67]. Polyphenols are found in a wide range of foods, including vegetables, fruits, tea, beans, and spices, and their consumption has been reported to improve metabolic syndrome (decreased body weight, decreased blood pressure, improved glucose metabolism, and improved lipid metabolism) [68]. However, up to 27% of ingested polyphenols are detected in urine [69], suggesting that many of them are not absorbed and reach the large intestine [70]. Since polyphenols reaching the large intestine have been reported to alter the proportions of microbiota [71], it is expected that the effect of polyphenols against metabolic syndrome is mediated through the improvement of dysbiosis and of the accompanying metabolic endotoxemia. There are two human intervention studies investigating the relationship between polyphenol intake and blood LPS, both of which evaluated the inhibitory effect on postprandial elevation of blood LPS levels (Table 1) [72,73]. In the study performed by Ghanim et al., healthy individuals ingested capsules containing 100 mg of resveratrol and 75 mg of polyphenol 10 min before a 930-kcal high-fat, high-carbohydrate meal. Blood LBP levels up to 5 h after a meal were evaluated and showed increased blood LBP levels in the placebo group but not in the capsule group [72]. On the other hand, Clemente-Postigo et al. administered 272 mL of red wine to humans simultaneously with excessive fat and found no effect on either blood LPS or LBP levels [73].

The efficacy of polyphenols has been also reported in animal studies. The effects of grape seed proanthocyanin [29,33], resveratrol [74], apple-derived polymeric procyanidins [75], genistein [76], isoflavone [77], and syringaresinol [78] on blood LPS levels in animal models have been reported (Table 3). In particular, L'openz et al. reported that six-month administration of genistein to high-fat diet-fed mice reduced their blood LPS levels and improved their spatial memory ability [76]. Cho et al. administered syringaresinol to 40-week-old mice for 10 weeks and showed that the decrease in blood LBP levels was accompanied with suppression of changes in immune cells due to aging (decreased naive T cells and decreased T-cell proliferation) [78]. It has also been reported that adoption of a high-fat diet results in abnormal differentiation of bone marrow hematopoietic stem cells due to increased blood LPS levels [79], suggesting that the effect of syringaresinol on immunoaging might be also exerted in other models of metabolic endotoxemia.

### 3.4. Sulfated Polysaccharide

Sulfated polysaccharides are widely present in animal tissues and seaweed and are used industrially as anticoagulants, pharmaceuticals, and gelling agents for foods.

The effect of sulfated polysaccharides on metabolic endotoxemia has been studied only in animals (Table 3). Intervention studies with sea cucumber-derived sulfated polysaccharides [80,81], acaudina molpadioides-derived fucosylated chondroitin sulfate [82], chicken-derived chondroitin sulfate [83] or fucoidan [84] have been performed. Of these studies, two showed that administration of sulfated polysaccharides to high-fat diet-fed mice increased

the amount of short-chain fatty acids in the intestinal tract, decreased the blood LPS or LBP concentration and attenuated weight gain [80,82]. Zhu et al. also reported the same effect of sulfated polysaccharides in chow-fed lean mice [81]. Liu et al. demonstrated that exhaustive exercise with a treadmill significantly impaired kidney function, decreased fecal butyrate levels, changed intestinal morphology, and induced metabolic endotoxemia [83]. Their study is interesting in showing that exercise stress also increased blood LPS levels, and that dietary factors are also effective in the model mice.

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3.5. Other Dietary Components/Extracts/Foods In the study by Abboud et al., obese or overweight subjects ingested 30 g of glutamine per day for eight weeks (Table 1) [85].

As a result, their blood LPS levels and waist circumference decreased. In an epidemiological study conducted with healthy subjects, 25-hydroxy vitamin D was reported to negatively correlate to blood LPS levels (Table 2) [86]. The protective effect of vitamin D is supported by animal studies in which vitamin D-deficient mice, exposed to a bacterial pathogen, exhibited lower LPS detoxification activity of the intestine and greater endotoxin translocation [87]. The effect of other dietary components, including tetrahydro iso-alpha acid [88], rhein [89], phlorizin [90], capsaicin [91], rutin [92], and lycopene [93] on blood LPS levels in animals has also been reported (Table 3). Among them, administration of tetrahydro iso-alpha acid [88], phlorizin [90], or rutin [92] to high-fat diet-fed mice or db/db mice improved metabolic impairment. Administration of rhein [89], or lycopene [93] to high-fat diet-fed mice showed a unique effect; they not only reduced blood LPS levels but also prevented high-fat diet-induced memory impairment. Kang et al. showed that administration of antibiotics to mice given capsaicin abolished the effect of capsaicin on blood LPS levels [91]. They also showed that capsaicin-induced protection against high-fat diet-induced blood LPS increase is transferrable by fecal microbiota transplantation. It has also been reported that intervention with crude food extracts or the food itself can lower blood LPS levels in animals (Table 3). We studied the effect of broccoli sprout extract, enriched in

functional glucosinolate "glucoraphanin" (details are described in Section 4) [94]. Anhê et al. examined the effects of extracts from cranberry [95] or camu camu [96]. Camu camu is an Amazonian fruit that contains an abundance of vitamin C and flavonoids such as ellagic acid, ellagitannins, and proanthocyanidins. Administration of camu camu extracts to high-fat/high-sucrose diet-fed mice reduced plasma bile acid pool size, altered gut microbiota composition, and reduced blood LPS levels. Dey et al. reported that administration of green tea extract to high-fat diet-fed mice suppressed inflammation and gut permeability especially in the ileum and colon, and reduced LPS influx from the portal vein [34]. The reduction of blood LPS levels by feeding with Tartary buckwheat protein was reported by Zhou et al. [32].

This study is valuable in that it elucidates one of the underlying mechanisms by which plant protein intake leads to improvement of metabolic abnormalities. Intervention studies with cocoa [97], nopal [98], and steamed fish meat [99] have been performed.

Among these, Zhan et al. performed unique experiments [99]. They divided mice into four groups, and fed them ad libitum with normal chow, steamed fish, pork or beef at 9:00 and 18:00 daily for eight weeks. As a result, only mice group fed with steamed fish showed decreased blood LBP levels compared to the other three groups.

3.6. Chinese Medicines The effect of the Chinese medicines; geniposide + chlorogenic acid [100], potentilla discolor bunge water extract [101], ganoderma lucidum mycelium water extract [102], semen hoveniae extract [103], and shenling baizhu powder [104] on blood LPS levels have been reported in animals (Table 3). The combination of geniposide and chlorogenic acid is included in a traditional Chinese medicine, Qushi Huayu Decoction. Peng et al. indicated that administration of geniposide and chlorogenic acid to high-fat diet-fed mice restored colon tight junctions by inhibiting down-regulation of RhoA/Rho-associated kinase signaling, and reduced blood LPS levels and hepatic LBP protein levels [100]. Han et al. examined the effect of potentilla discolor bunge water extract in type 2 diabetic mice induced by high-fat diet feeding and streptozotocin injection [101]. The results showed that fecal LPS levels in the type 2 diabetic model mice were significantly increased compared to the control normal mice. The administration of potentilla discolor bunge water extract to mice reduced fecal LPS levels, decreased blood LPS levels and increased the expression levels of tight junction proteins (Claudin-3, ZO-1, and Occludin) in the colon. Chang et al. studied the effect of ganoderma lucidum mycelium, a Basidiomycete fungus [102]. They showed the dose-dependent effect of ganoderma lucidum mycelium water extract on blood LPS reduction, suggesting that high molecular weight polysaccharides (>300 kDa) isolated from the extract is an effective component. Ping et al. reported that the extract of

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semen hoveniae, a seed of *Hovenia dulcis* Thunb rich in dihydromyricetin and quercetin, decreased blood LPS levels in a mouse model of alcohol-induced liver injury [103]. It has been reported that administration of shenling baizhu, a mixture of ten different traditional Chinese medicinal herbs, to high-fat diet-fed mice decreased LPS levels in the portal vein [104].

3.7. Dietary Habits In relation to dietary habits, Kopf et al. conducted an intervention study in humans with BMI > 25 kg/m<sup>2</sup> and low intake of whole grains, fruits, and vegetables (Table 1) [65]. During the weekly interview, the subjects themselves selected the vegetables and fruits to be eaten the following week from apples, bananas, blueberries, clementines, grapes, pears, strawberries, broccoli, carrots, cauliflower, celery, green beans, green leaf lettuce, peas, spinach, sweet pepper, and tomatoes. The subjects ate these fruits and vegetables for 21 to 30 servings/week (at least three servings/day) for six weeks. As a result, compared to control group in that dietary habits were not or minimal changed, average daily intake of refined grains was 1/3, fruit intake was doubled, and vegetable intake was four times, leading to a significant reduction in blood LBP levels and IL-6 levels. An epidemiological study by Ahola et al. in patients with type 1 diabetes has shown a negative correlation between several dietary patterns and blood LPS levels: These dietary patterns are “Fish” (frequently eats fish dishes), “Healthy snack” (frequently eats fruits, berries, fresh vegetables, yoghurt, low-fat cheese, and does not drink many soft drinks) and “Modern” (frequently eats poultry, pasta, rice, meat dishes, fried and grilled foods, and fresh vegetables) (Table 2) [105]. In the epidemiological study by Ahola et al., no significant correlation was found between blood LPS levels and intake of energy, carbohydrates, fats, proteins, or dietary fiber. In regard to the absence of a significant positive correlation between blood LPS levels and fat intake (the believed cause of blood LPS elevation in humans and animals), the authors consider the

the previously reported amount or proportion of fat intake may be greater than the intake in the normal diet. Similarly, Amar et al. reported no significant correlation between fat intake and blood LPS levels in 201 subjects [106]. In the same study, Amar et al. reported a positive correlation between total energy intake and blood LPS levels [106]. The effect of caloric restriction on blood LPS levels have been reported in both humans and mice. Ott et al. reported that, in women with a BMI of 30 kg/m<sup>2</sup> or more, intake of a defined formula diet of 800 kcal/day for four weeks decreased blood LBP levels, and following intake of the normal diet (1800 kcal/day), blood LBP levels returned to the initial levels (Table 1)[107]. Even in mice, caloric restriction of 30% [108] or 40% [109] has been reported to decrease blood LPS or LBP levels (Table 3). A common finding in these reports in mice is that blood LPS or LBP levels are reduced by calorie restriction compared to ad libitum even in normal chow-fed mice. This suggests that the influx of LPS into the bloodstream is not limited to the specific conditions of excessive fat intake but can also occur by some mechanism in the normal diet.

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Table 1. Dietary factors that have been evaluated for efficacy on blood lipopolysaccharide (LPS) levels in human interventional studies.

Category Dietary Factor Dose Consumption Period

Subject LPS LBP

Gut Microbes with Significant Changes in Proportion \*\* Increase Decrease

Probiotics/ Prebiotics

Yakult light (Lactobacillus casei Shirota 1×10<sup>8</sup> CFU/mL) [48]

195 mL 3 months Metabolic syndrome ND ↑ — —

Low-fat yogurt [49] 339 g 9 weeks Healthy subject or Obesity → → — —

Low-fat yogurt [50] 226 g Premeal

Healthy subject or Obesity (postprandial endotoxemia was assessed) → → — —

Oligofructose [57] 21 g 12 weeks Overweight/ Obesity ↓ — — — Oligofructose enriched

inulin [58] 10 g 8 weeks Type 2 diabetes ↓ — — —

Inulin + Oligofructose [59]

8 g 8 g 3 months Obesity → —

Bifidobacterium, Faecalibacterium prausnitzii

Bacteroides intestinalis, Bacteroides vulgatus, Propionibacterium

Galactooligosaccharide [60]

5.5 g 12 weeks Type 2 diabetes → → none none

Galactooligosaccharide [61]

15 g 12 weeks Overweight/ Obesity — → Bifidobacterium spp. none

α-Galactooligosaccharide [62]

6–18 g 14 days Overweight ↓ — Bifidobacteria none

Resistant dextrin [63] 10 g 8 weeks Type 2 diabetes ↓ — — — Insoluble dietary fiber [from Fiber One Original cereal (General mills)] [64] 30 g With high-fat, high-calorie meal Healthy

subject (postprandial endotoxemia was assessed) ↓\* — — — Whole grains [65] 3 servings

6 weeks Overweight/ Obesity — ↓ none none

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Table 1. Cont.

Category Dietary Factor Dose Consumption Period

Subject LPS LBP

Gut Microbes with Significant Changes in Proportion \*\* Increase Decrease

Probiotics/ Prebiotics

Bifidobacterium longum + Oligofructose + Life style modification [110]

— 24 weeks Non-alcoholic steatohepatitis ↓ — — —

Polyphenol

Resveratrol + Polyphenol [72]

100 mg 75 mg

10 minutes before intake of high-fat high-carbohydrate meal

Healthy subjects (postprandial endotoxemia was assessed)

— ↓\* — —

Red wine [73] 272 mL With high-fat meal

Healthy subjects (postprandial endotoxemia was assessed) → → — —

Dietary habits

Fruits + Vegetables [65]

3 servings 6 weeks Overweight/ Obesity — ↓

α-diversity (No significant change in bacterial genera was found)

none

Caloric restriction [107] 800 kcal 4 weeks Obesity — ↓

Anaerostipes hadrus, Blautia sp., Ruminococcus faecis, Bifidobacterium sp.

Agathobacter rectalis

Others Glutamine [85] 30 g 14 weeks Overweight/ Obesity ↓ — — — ND: Not detected, —:

No data,↑: Significantly increased,→: Not significantly changed,↓: Significantly decreased, \*:

Attenuation of postprandial endotoxemia, \*\*: The bacteria mentioned by the author in the paper are listed.

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Table 2. Correlation of dietary factors, gut microbes, and blood LPS levels in human epidemiological studies.

Subject Number of Subject

Correlation of Dietary Factor and Gut Microbe \*

Correlation of Blood LPS and Gut Microbe

Correlation of Blood LPS and Dietary Factor

Overweight pregnant women [111]

88

P

Dietary fiber vs.

diversity, richness, Firmicutes in unidentified family of order Clostridiales, Barnsiellaceae family belonging to the phylum Bacteroidetes

P none P none

Vitamin A, β-Carotene vs.

Firmicutes

N Fat vs. diversity, richness, Barnsiellaceae N none N none

Healthy subjects [86]

150 N

25-Hydroxy vitamin D vs.

Coprococcus, Bifidobacterium N LPS vs. Faecalibacterium N LPS vs. 25-Hydroxy vitamin D

Type 1 diabetes [105]

668 — — — — — N LPS vs.

Dietary pattern; “Fish”(frequently eat fish dishes), “Healthy snack” (frequently eat fruits, berries, fresh vegetable, yoghurt, low-fat cheese, and do not drink much soft drinks), “Modern”(frequently eat poultry, pasta, rice, meat dishes, fried and grilled foods, and fresh vegetables) —: No data, P: Positive correlation, N: Negative correlation, LPS:

lipopolysaccharide, \*: The bacteria mentioned by the author in the paper are listed.

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Table 3. Dietary factors that have been evaluated for efficacy on blood LPS levels in animal interventional studies. Category Dietary Factor Dose Administration Period Model LPS LBP Significant Change in Gut Microbiota

Probiotics/ Prebiotics

Lactobacillus rhamnosus GG [51] 1×10<sup>8</sup> CFU/day 12 weeks HFD-fed ApoE KO mouse ↓ —  
no Lactobacillus rhamnosus CNCM I-4036 [52] 1×10<sup>10</sup> CFU/day 30 days Chow diet-fed Zucker-Lepfa/fa rat — ↓ — Lactobacillus sakei OK67 +/- Lactobacillus sakei PK16 [53] 1×10<sup>9</sup> CFU/day 1×10<sup>9</sup> CFU/day 4 weeks HFD-fed C57BL/6 mouse ↓ — yes  
Bifidobacterium longum BR-108 (sterilized) [54] 200, 400 mg/kg/day 4 weeks HFD-fed C57BL/6J mouse ↓ — yes Bifidobacterium infantis + Lactobacillus acidophilus + Bacillus cereus [55] 0.5×10<sup>6</sup> CFU/day 0.5×10<sup>6</sup> CFU/day 0.5×10<sup>5</sup> CFU/day 12 weeks HFHSD-fed SD rat ↓ — yes Lactobacillus plantarum LC27 +/- Bifidobacterium longum LC67 [43] 1×10<sup>9</sup> CFU/day each (or 0.75×10<sup>9</sup> (LC27) + 0.25×10<sup>9</sup> (LC67) CFU/day in mix) 4 weeks HFD-fed C57BL/6 mouse ↓ — yes Oligofructose [112] 10% (mixed in diet) 12 weeks HFHSD-fed SD rat ↓ — yes Galactooligosaccharide [84] 800 mg/kg/day 8 weeks HFD-fed SD rat ↓ — yes Inulin [113]

5% (intragastric administration, sample volume was not described)

6 weeks

standardized diet (kcal %: 10% fat, 20% protein, and 70% carbohydrate; 3.85 kcal g<sup>-1</sup>)-fed db/db mouse

↓ — yes

Wheat-derived arabinoxylan [114] 7.5% (mixed in diet) 8 weeks HFD-fed C57BL/6J mouse

↓ — —

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Table 3. Cont. Category Dietary Factor Dose Administration Period Model LPS LBP Significant Change in Gut Microbiota

Polyphenols

Grape seed proanthocyanidin [33] 500 mg/kg/day

10 days (prophylactic) or 17 weeks (with cafeteria diet)

Cafeteria diet (high-fat/high carbohydrate diet)-fed Wistar rat

↓ — —

Grape-seed proanthocyanidin [29] 100, 500 mg/kg/day 2 weeks

Cafeteria diet (high saturated-fat/high refined-carbohydrate diet)-fed Wistar rat

↓ — —

Resveratrol [74] 50, 75, 100 mg/kg/day 16 weeks HFD-fed C57BL/6 mouse ↓ ↓ yes Apple-

derived polymeric procyanidins [75] 0.5% (administration route was not described) 20

weeks HFHSD-fed C57BL/6J mouse ↓ — yes Genistein [76] 0.2% (mixed in diet) 6 months

HFD-fed C57BL/6 mouse ↓ — yes Isoflavone [77] 0.1% (mixed in diet) 5 weeks HFD-fed

C57BL/6 mouse ↓ ↓ yes Syringaresinol [78] 50 mg/kg/day 10 weeks 40-week-old C57BL/6 mouse — ↓ yes

Sulfated polysaccharide

Sea cucumber-derived sulfated polysaccharide [80]

300 mg/kg/day 8 weeks HFD-fed BALB/c mouse — ↓ yes

Sea cucumber-derived sulfated polysaccharide [81]

300 mg/kg/day 42 days Chow-fed BALB/c mouse — ↓ yes

Acaudina molpadioides-derived fucosylated chondroitin sulfate [82]

80 mg/kg/day 10 weeks HFD-fed C57BL/6J mouse ↓ — yes

Chicken-derived chondroitin sulfate [83]

150 mg/kg/day 16 days

Exhaustive exercise stress model BALB/c mouse ↓ — yes

Fucoidan [84] 100 mg/kg/day 8 weeks HFD-fed SD rat ↓ — yes

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Table 3. Cont. Category Dietary Factor Dose Administration Period Model LPS LBP

Significant Change in Gut Microbiota

Other dietary components

Tetrahydro iso-alpha acid (included in hops) [88]

0.1% (mixed in diet) 8 weeks HFD-fed C57BL/6J mouse ↓ — —

Rhein (included in rhubarb) [89] 120 mg/kg/day 6 weeks HFD-fed C57BL/6J mouse ↓ —

yes Phlorizin (included in apple) [90] 20 mg/kg/day 10 weeks Chow-fed db/db mouse ↓ —

yes Capsaicin [91] 0.01% (mixed in diet) 12 weeks HFD-fed C57BL/6J mouse ↓ — yes Rutin

[92] 0.64% (mixed in diet) 20 weeks HFD-fed C57BL/6J mouse ↓ — yes Lycopene [93]

0.03% (mixed in diet) 10 weeks HFD and fructose-fed C57BL/6 J mouse ↓ — —

Other extracts/dietary components

Broccoli sprout extract [94] 2.2% (mixed in diet) 14 weeks HFD-fed C57BL/6JSlc mouse ↓ ↓

yes Camu camu extract [96] 200 mg/kg/day 8 weeks HFHSD-fed C57BL/6J mouse ↓ — yes

Other extracts/dietary components

Cranberry extract [95] 200 mg/kg/day 8 weeks HFHSD-fed C57BL/6J mouse ↓ — yes

Green tea extract [34] 2% (mixed in diet) 8 weeks HFD-fed C57BL/6J mouse ↓ — yes

Tartary buckwheat protein [32] 23.5% (mixed in diet) 6 weeks HFD-fed C57BL/6 mouse ↓

— yes

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Table 3. Cont. Category Dietary Factor Dose Administration Period Model LPS LBP

Significant Change in Gut Microbiota

Foods

Cocoa [97] 8% (mixed in diet) 18 weeks HFD-fed C57BL/6J mouse ↓ — —

Nopal [98]

5% of dietary fiber was replaced with those of nopal-derived (mixed in diet)

1 month HFHSD-fed Wistar rat ↓ — yes

Steamed fish meat [99]

Ad libitum (9:00–12:00 and 18:00–21:00)

8 weeks Chow-fed C57BL/6 mouse — ↓ yes

Chinese medicines

Geniposide + Chlorogenic acid [100]

90 mg/kg/day 1.34 mg/kg/day 4 weeks HFD-fed C57BL/6 mouse — ↓ —

Potentilla discolor Bunge water extract [101]  
 400 mg/kg/day 8 weeks  
 HFD-fed, streptozotocin-injected C57BL/6J mouse ↓ ↓ yes  
 Ganoderma lucidum mycelium water extract [102]  
 2–8 mg/day 8 weeks HFD-fed C57BL/6NCrlBlw mouse ↓ — yes  
 Semen hoveniae extract [103] 300, 600 mg/kg/day 8 weeks  
 Alcohol-containing Lieber-DeCarli diet-fed SD rat (Alcoholic liver disorder model)  
 ↓ — yes

Shenling Baizhu powder [104] 30 g/kg/day 16 weeks HFD-fed SD rat ↓ — yes  
 Caloric restriction

30% caloric restriction [108] — 62–141 weeks HFD, LFD-fed C57BL/6J mouse — ↓ yes  
 40% caloric restriction [109] — 30 days Chow-fed C57BL/6J mouse ↓ ↓ yes —: No data,  
 HFD: High-fat diet, HFHSD: High-fat high-sucrose diet, ↑: Significantly increased, →: Not  
 significantly changed, ↓: Significantly decreased.

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#### 4. Association of Dietary Factor-Induced Reduction of Blood LPS and Modulation of Gut Microbiota

Although few studies have evaluated the relationship between the effect of dietary factors on blood LPS and intestinal flora in humans, several studies have evaluated intestinal flora in oligosaccharide intervention studies (Table 1). A common finding in these reports is an increase in Bifidobacterium. Bifidobacterium has been reported to enhance the intestinal tight junction by preserving claudin 4 and occludin localization at tight junctions, and inhibit permeability in mice with colitis [115]. Similarly, in human colonic epithelial cell line T84, the addition of culture supernatant of Bifidobacterium has been reported to enhance barrier function through increased expression of tight junction protein, suggesting that some humoral factors contribute to improved intestinal barrier function [116]. Increased expression of tight junction protein in Bifidobacterium-treated mice has been reported to be associated with increased short-chain fatty acids (acetic acid, butyric acid, and propionic acid) in the intestinal tract [117]. These short-chain fatty acids have been reported in the human colonic epithelial cell line caco-2 to act as an energy source for epithelial cells to protect themselves, and also act as a histone deacetylase inhibitor which inhibit Nod-like receptor P3 inflammasomes to maintain the barrier function of epithelial cells [118]. These results suggest that the increase in Bifidobacterium induced by oligosaccharide intake decreases blood LPS levels through the improvement of the barrier function of the intestinal tract. In addition, dietary factors that increase Bifidobacterium are expected to reduce blood LPS levels. Changes in intestinal flora by the dietary factors listed in Table 4 was greatly dependent on the study. However, all of the dietary factors commonly lowered blood LPS or LBP levels in animals, as described in Table 3. In other words, by finding bacteria that have decreased or increased in many dietary factor intervention studies, we can find specific bacteria that contribute to the increase or decrease in blood LPS levels. To this end, we have organized the number of reports that show increases or decreases of each bacterial genus (Figure 1).

We selected eight of these genera (Lactobacillus, Bacteroides, Akkermansia, Clostridium, Escherichia, Roseburia, Prevotella and Desulfovibrio) as bacteria included in a sufficient number (five or more) of reports, and a biased number of reports (Bifidobacterium was excluded because it was discussed above. Faecalibacterium was also excluded

because there is almost no bias in the number of reports). *Lactobacillus*, *Bacteroides*, *Akkermansia*, *Roseburia*, and *Prevotella* are possible bacterial genera that may contribute to the reduction of blood LPS levels. *Lactobacillus* is a gram-positive bacterium that produces large amounts of lactic acid during carbohydrate fermentation. The probiotic contribution of *Lactobacillus* to the regulation of metabolic endotoxemia is studied (Table 3). Administration of *Lactobacillus rhamnosus* CNCM I-4036 to obese Zucker-Leprfa/fa rats decreased the mRNA expression levels of endothelin receptor type B (*Ednrb*) in the intestinal mucosa, and reduced the blood LBP level [52]. Reduction of *Ednrb* decreases the density of negative charge of the colonic mucin layer, leading to an increase in the ability of the mucin layer to adsorb microparticles and bacteria, thereby inhibiting their penetration through the colonic mucosa [119]. *Lactobacillus sakei* OK67 and PK16 are reported to suppress high-fat diet-induced colitis, and to reduce the fecal Proteobacteria population and fecal LPS levels in mice [53]. In addition to the previous reports described in Table 3, it has been reported that oral administration of *Lactobacillus reuteri* ZJ617 suppresses LPS-induced apoptosis of intestinal epithelial cells and maintains the intestinal barrier function [120]. We have described in Section 2.2 that LPS is absorbed from the intestinal tract during lipid absorption. Interestingly, oral ingestion of *Lactobacillus acidophilus* ATCC 4356 in mice has been reported to reduce the mRNA levels of Niemann-Pick C1-like 1, which is involved in lipid absorption in the intestine, and in the suppression of cholesterol absorption [121]. Taken together, this suggests that *Lactobacillus* contributes to a decrease in blood LPS levels through strengthening the intestinal barrier, reducing the amount of LPS in feces, and suppressing lipid absorption. As described in Table 4, Bifidobacterium, oligofructose, galacto-oligosaccharide, syringaresinol, *acaudina molpadioides*-derived fucosylated chondroitin sulfate, green tea extract, Tartary buckwheat protein, nopal, semen hoveniae extract,

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and 30% caloric restriction are dietary factors that increase the proportion of *Lactobacillus* in the gut microbiota. Among them, the amyolytic Bifidobacterium strain is reported to stimulate the growth of a

nonamyolytic *Lactobacillus* probably by producing intermediate metabolites of starch metabolism [122]. Oligosaccharides (oligofructose and galacto-oligosaccharide) were reported to support the growth of *Lactobacillus* as prebiotics [123]. Green tea extract [124] and buckwheat-resistant starch [125] were reported to promote the growth of *Lactobacillus* in a fermentation assay. On the other hand, in an in vitro fermentation assay using gut microbiota, it was reported that fucosylated chondroitin sulfate promotes the growth of *Bacteroides*, Bifidobacterium, and *Clostridium*, while the number of *Lactobacillus* decreases [126]. Thus, the mechanism by which *Lactobacillus* increased in mice fed with fucosylated chondroitin sulfate needs to be further studied.

The mechanism by which the proportion of

*Lactobacillus* in gut microbiota increases due to caloric restriction also remains unknown.

As it has been reported that the bacteria adapted to the nutritional environment can grow predominantly in the gut

microbiota consortium [127], *Lactobacillus* might be able to grow even under malnutrition.

The effect of syringaresinol, nopal, and semen hoveniae on the growth of *Lactobacillus* has not been revealed. *Bacteroides* is a gram-negative obligate anaerobe. Hooper et al. reported that *Bacteroides thetaiotaomicron*, a prominent component of the normal mouse and

human intestinal microflora, modulates expression of genes involved in mucosal barrier fortification [128]. The administration of *Bacteroides fragilis* HCK-B3 and *Bacteroides ovatus* ELH-B2 to mice attenuated LPS-induced intestinal inflammation, by either modulating cytokine production or restoring the Treg/Th-17 balance [129]. On the other hand, in a state in which no dietary fiber is ingested, it has been suggested that *Bacteroides* degrades the mucin layer of the intestinal tract, decreases the barrier function of mucus, and induces inflammation [130]. Therefore, it should be noted that depending on the diet of the host, *Bacteroides* can act as either probiotics or pathobionts. As described in Table 4, an increase in *Bacteroides* was reported in four out of five intervention studies with sulfated polysaccharides. *Bacteroides* is a unique bacterium among gut flora that has degrading enzymes corresponding to various sulfated polysaccharides [131] and is able to utilize sulfated polysaccharides such as heparin [131], heparan sulfate [131], and chondroitin sulfate [132] as energy sources. It is therefore thought that intake of sulfate polysaccharide preferentially nourishes *Bacteroides* in gut flora and suppresses metabolic endotoxemia via its anti-inflammatory and barrier function-enhancing effects. *Akkermansia* is a mucin-adherent intestinal bacterium [133], which grows by degrading mucin [134], and produces propionic acid, a short-chain fatty acid [135].

In addition, *Akkermansia* promotes butyrate production, by supporting the growth of *Anaerostipes caccae* through mucin degradation [136]. As noted above, these short-chain fatty acids are known to enhance intestinal barrier function. In addition, it has been reported that *Akkermansia*-derived extracellular vesicles administered in mice are localized to the large intestine, and directly enhance intestinal barrier function by increasing epithelial cell expression of tight junction proteins [137].

Furthermore, oral administration of *Akkermansia* to mice inhibited high-fat diet-induced thinning of the mucin layer, reduced blood LPS concentration, and inhibited obesity and abnormal glucose metabolism [138]. *Akkermansia* has been reported to be negatively correlated with obesity (waist-to-hip ratio and subcutaneous adipocyte diameter) and diabetes mellitus (glucose intolerance states), and is attracting attention as a next-generation probiotic [139]. Among the dietary factors that increase the proportion of *Akkermansia* in the gut flora, polyphenols are intriguing because most of intervention studies with polyphenols (apple-derived polymeric procyanidins, genistein, and isoflavone) or polyphenol-rich food extracts (camu camu extract, cranberry extract, and green tea extract) consistently reported an increase of *Akkermansia* (Table 4). Anhê et al. reported that cranberry extract administration to mice increased colonic Kruppel-like factor 4 (a marker of goblet cells) and Muc2 mRNA expression, suggesting that polyphenols enhance mucin production and support the growth of *Akkermansia* [95].

On the other hand, direct prebiotic action of polyphenols to *Akkermansia* has been reported in a study using the Simulator of Human Intestinal Microbial Ecosystem (SHIME®) [140].

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Table 4. Changes of gut microbiota induced by dietary factor intervention in animal experiments.

Category Dietary Factor Sample Method

Gut Microbe with Significant Changes in Proportion \* Increase Decrease

Probiotics/ Prebiotics

Lactobacillus sakei OK67 +/- Lactobacillus sakei PK16 [53]  
 Feces PCR, NGS OTU (O67), Ace (O67), Chao1 (O67), Shannon (O67)  
 Simpson (O67), Proteobacteria, Firmicutes, Firmicutes/Bacteroidetes,  
 Proteobacteria/Bacteroidetes  
 Bifidobacterium longum BR-108 (sterilized) [54]  
 Cecal contents PCR Bifidobacterium spp., Lactobacillus spp. Firmicutes  
 Bifidobacterium infantis + Lactobacillus acidophilus + Bacillus cereus [55]  
 Feces PCR  
 Bifidobacteria, Lactobacillus, Bacteroides, Bifidobacteria/Escherichia coli  
 Escherichia coli, Enterococcus  
 Lactobacillus plantarum LC27 +/- Bifidobacterium longum LC67 [43]  
 Feces PCR Actinobacteria (LC67, LC27 + LC67)  
 Firmicutes, Bacteroidetes,  $\delta/\gamma$ -Proteobacteria, Deferribacteres (LC67, LC27+LC67),  
 Firmicutes/Bacteroidetes, Proteobacteria/Bacteroidetes  
 Oligofructose [112] Cecal contents PCR  
 Bacteroides/Prevotella, Bifidobacterium, Lactobacillus, Roseburia  
 Clostridium leptum (cluster IV), Clostridium cluster I, Clostridium cluster XI,  
 Methanobrevibacter, Akkermansia muciniphila, Faecalibacterium prausnitzii  
 Galacto-oligosaccharide [84] Cecal contents NGS  
 Verrucomicrobia, Akkermansia, Ruminococcus, Blautia, Bacteroidetes, Proteobacteria,  
 Adlercreutzia, Staphylococcus, Prevotella, Oscillospira, Lactobacillus, Desulfovibrio  
 Firmicutes, Actinobacteria, Clostridium, Bacillus  
 Inulin [113] Feces NGS Bacteroidetes, Cyanobacteria, Bacteroides  
 Firmicutes, Deferribacteres, Tenericutes, Ruminiclostridium\_6, Mucispirillum  
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 Table 4. Cont.  
 Category Dietary Factor Sample Method  
 Gut Microbe with Significant Changes in Proportion \* Increase Decrease  
 Polyphenols  
 Resveratrol [74] Cecal contents NGS Deferribacteraceae  
 none (In this study, population of Desulfovibrionaceae in the high-fat diet + intervention  
 group was at the same level with normal chow group, but there was no significant  
 reduction from high-fat diet group.)  
 Apple-derived polymeric procyanidins [75]  
 Cecal contents NGS  
 Bacteroidetes, Verrucomicrobia, Adlercreutzia, Roseburia, S24-7, Bacteroids, Anaerovorax,  
 rc4-4, Akkermansia  
 Firmicutes, Firmicutes/Bacteroidetes, Clostridium, Lachnospiraceae, Bifidobacterium  
 Polyphenols  
 Genistein [76] Feces NGS  
 Firmicutes, Verrucomicrobia, Prevotellaceae, Verrucomicrobia, Prevotella, Akkermansia,  
 Faecalibacterium, Prevotella copri, Prevotella stercorea, Akkermansia muciniphila  
 Bacteroidetes, Bacteroidaceae, Bacteroides, Bacteroides acidifaciens, Bacteroides uniformis  
 Isoflavone [77] Feces NGS  
 $\alpha$ -diversity, Actinobacteria, Verrucomicrobia, Bifidobacterium/Enterobacteriaceae,  
 Akkermansia

Proteobacteria

Syringaresinol [78] Cecal contents NGS

Firmicutes/Bacteroidetes, Firmicutes, Lactobacillus, Lactobacillus animalis, Lactobacillus johnsonii, Lactobacillus reuteri, Lactobacillus intestinalis, Bifidobacterium pseudolongum Shannon diversity indices, Jeotgalicoccus nanhaiensis, Staphylococcus lentus, Bacteroidaceae (EF098405\_s), Bacteroides vulgatus, Akkermansia muciniphila  
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Table 4. Cont.

Category Dietary Factor Sample Method

Gut Microbe with Significant Changes in Proportion \* Increase Decrease

Sulfated polysaccharide

Sea cucumber-derived sulfated polysaccharide [80]

Feces NGS

bacterial diversity, Verrucomicrobia (depolymerized sulfated polysaccharide), Bacteroides, Alloprevotella, Ruminiclostridium\_9, Butyricoccus, Akkermansia Proteobacteria, Escherichia-Shigella (polymerized sulfated polysaccharide), Pseudomonas (depolymerized sulfated polysaccharide), Yersinia (depolymerized sulfated polysaccharide), (In this study, decrease of Desulfovibrio with the intervention of sulfated polysaccharide to high-fat diet-fed mouse was shown as heatmap, but significance of difference was not described.)

Sea cucumber-derived sulfated polysaccharide [81]

Feces NGS

Proteobacteria (polymerized sulfated polysaccharide), Bacteroides (polymerized sulfated polysaccharide), Allobaculum (depolymerized sulfated polysaccharide), Alloprevotella, Roseburia, Turicibacter, Desulfovibrio

Enterococcus, Streptococcus, Escherichia-Shigella, Lactobacillus

Acaudina molpadioides-derived fucosylated chondroitin sulfate [82]

Feces PCR, NGS

Bacteroidetes, Lactobacillus, Actinobacteria, Faecalibacterium prausnitzii, Deferribacteres, Bacteroidales, Bifidobacteriales, Lachnospiraceae NK4A136 group, Bacteroides, Bacteroides acidifaciens, Bifidobacterium choerinum

Firmicutes, Escherichia coli, Clostridiales, Bacilli, Lactobacillales, Clostridia Clostridiales,

Firmicutes Clostridiales, Lactococcus, Clostridium ruminantium

Chicken-derived chondroitin sulfate [83]

Feces NGS

Bacteroidetes, Bacteroides acidifaciens, family S24-7, Lysinibacillus boronitolerans

Firmicutes,  $\beta$ -Proteobacteria

Furoidan [84] Cecal contents NGS

Proteobacteria, Verrucomicrobia, Enterobacter, Bacteroidetes, Bacillus, Ruminococcus,

Adlercreutzia, Prevotella, Oscillospira, Desulfovibrio,

Firmicutes, Actinobacteria, Clostridium, Corynebacterium, Staphylococcus, Lactobacillus,

Aerococcus

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Table 4. Cont.

Category Dietary Factor Sample Method

Gut Microbe with Significant Changes in Proportion \* Increase Decrease

Other dietary components

Rhein (included in rhubarb) [89]

Cecal contents PCR Bacteroides/Prevotella, Desulfovibrio Bifidobacterium, Lactobacillus Phlorizin (included in apple) [90]

Feces PCR, DGGE Akkermansia muciniphila, Prevotella none

Capsaicin [91] Cecal contents NGS Ruminococcaceae, Lachnospiraceae family S24\_7

Rutin [92]

Small intestinal contents NGS

Bacteroidales\_S24-7 group, Bacteroidaceae, Porphyromonadaceae, Rikenellaceae, Desulfovibrionaceae

Firmicutes, Firmicutes/Bacteroidetes, Deferribacteraceae, Lachnospiraceae

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Table 4. Cont.

Category Dietary Factor Sample Method

Gut Microbe with Significant Changes in Proportion \* Increase Decrease

Other extracts/dietary components

Broccoli sprout extract [94] Cecal contents NGS none Proteobacteria, Desulfovibrionaceae

Camu camu extract [96] Feces NGS

microbial richness, Bifidobacterium, Barnesiella, Barnesiella spp., Turicibacter spp., Akkermansia muciniphila, Delftia, Roseburia, Anaerostipes, unclassified genera within the families Christensenellaceae, unclassified genera within the families Erysipelotrichaceae

Firmicutes/Bacteroidetes, Lactobacillus, Anaerotruncus, Parabacteroides

Cranberry extract [95] Feces PCR, NGS Akkermansia none

Green tea extract [34] Cecal contents NGS

Shannon index, Chao1 richness, Bacteroidetes, Actinobacteria, Verrucomicrobia,

Bacteroidales, Bifidobacteriales, Verrucomicrobiales, Turicibacterales. RF39,

Coriobacteriales, Bifidobacterium, Blautia, Dorea, Lactobacillus, Ruminococcus,

Akkermansia, Butyrivibrio, Akkermansia muciniphila, Ruminococcus gnavus,

Bifidobacterium pseudolongum, Bifidobacterium adolescentis

Firmicutes, Firmicutes/Bacteroidetes, Clostridiales, SMB53

Tartary buckwheat protein [32]

Feces PCR Bifidobacterium, Lactobacillus, Enterococcus, Clostridium Escherichia coli, Bacteroides

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Table 4. Cont.

Category Dietary Factor Sample Method

Gut Microbe with Significant Changes in Proportion \* Increase Decrease

Foods

Nopal [98] Feces NGS

$\alpha$ -diversity, Anaeroplasma, Prevotella, Ruminococcus, Bacteroides fragilis, Ruminococcus bromii, Ruminococcus flavefaciens, Lactobacillus reuteri, Akkermansia muciniphila

Firmicutes/Bacteroidetes, Faecalibacterium, Clostridium, Butyricoccus, Bacteroides

acidifaciens, Blautia producta, Faecalibacterium prausnitzii, Butyricoccus pullicaecorum, Clostridium citroniae

Steamed fish meat [99] Feces NGS

Proteobacteria, Firmicutes, Ruminococcaceae, Oscillospira, Clostridium, Escherichia

Shannon index, Bacteroidetes, S24-7

Chinese medicines

Potentilla discolor Bunge water extract [101]

Feces NGS

Bacteroidetes, Bacteroidales\_S24-7\_group, norank\_f\_Bacteroidales\_S24-7\_group, Parabacteroides, Eubacterium\_nodatum\_group, norank\_f\_Rhodospirillaceae, Tyzzerella, Rikenella, Alistipes, Lachnospiraceae\_NK4A136\_group, norank\_f\_Ruminococcaceae, Romboutsia, Coriobacteriaceae\_UCG\_002, Bacteroides, Allobaculum, Coprococcus\_3, norank\_f\_Christensenellaceae

Proteobacteria, Helicobacteraceae, Helicobacter

Ganoderma lucidum mycelium water extract [102]

Cecal contents NGS

Parabacteroides goldsteinii, Bacteroides spp., Anaerotruncus colihominis, Roseburia hominis, Clostridium methylpentosum (Clostridium IV), Clostridium XIVa, Clostridium XVIII, Eubacterium coprostanoligenes

Firmicutes/Bacteroidetes, Proteobacteria, Mucispirillum shaedleri, Escherichia fergusonii, Enterococcus spp., Lactococcus lactis, Clostridium lactatifermentans (Clostridium XIVb), Oscillibacter valericigenes

Semen hoveniae extract [103] Feces NGS

Shannon index, Verrucomicrobia, Bacteroidetes, Parabacteroides, Alloprevotella, Alistipes, Lactobacillus, Akkermansia

Proteobacteria, Firmicutes/Bacteroidetes, Oscillibacter, Helicobacter

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Table 4. Cont.

Category Dietary Factor Sample Method

Gut Microbe with Significant Changes in Proportion \* Increase Decrease

Chinese medicines

Shenling Baizhu powder [104] Feces NGS

Shannon index, Actinobacteria, Cyanobacteria, Anaerostipes, Bifidobacterium

Firmicutes/Bacteroidetes, Blautia, Roseburia, Phascolarctobacterium, Desulfovibrio (Significance of difference was not described)

Caloric restriction

30% caloric restriction [108] Feces NGS

(low-fat diet vs. low-fat diet with caloric restriction) Lactobacillus, OTU45 (in Lactobacillus), Bifidobacterium, [increased by caloric restriction with both of low-fat diet or high-fat diet] OTU119, OTU155, OTU267 (in Tannerella)

(low-fat diet vs. low-fat diet with caloric restriction) Streptococcaceae, TM7, OTU469 (in Desulfovibrionaceae) [decreased by caloric restriction with both of low-fat diet or high-fat diet] OTU65 (in Lactococcus), OTU366 (in Bacteroidales), OTU37 (in

Peptostreptococcaceae),

40% caloric restriction [109] Feces NGS Lactobacillaceae, Erysipelotrichaceae,

Bacteroidaceae, Verrucomicrobiaceae Firmicutes PCR: Polymerase chain reaction, NGS:

Next-generation sequencing, DGGE: Denaturing gradient gel electrophoresis, OUT:

Operational taxonomic unit, \*: The bacteria mentioned by the author in the paper are listed.

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Figure 1. The number of reported changes of intestinal bacterial genera in dietary factor intervention studies in animals.

Lactobacillus, Bacteroides, Akkermansia, Roseburia, and Prevotella are possible bacterial genera that may contribute to the reduction of blood LPS levels. Lactobacillus is a gram-positive bacterium that produces large amounts of lactic acid during carbohydrate fermentation. The probiotic contribution of Lactobacillus to the regulation of metabolic endotoxemia is studied (Table 3). Administration of Lactobacillus rhamnosus CNCM I-4036 to obese Zucker-Leprfa/fa rats decreased the mRNA expression levels of endothelin receptor type B (Ednrb) in the intestinal mucosa, and reduced the blood LBP level [52]. Reduction of Ednrb decreases the density of negative charge of the colonic mucin layer, leading

8 6 4 2 0 2 4 6 8 10 12

Lactobacillus Bacteroides Akkermansia Bifidobacterium Clostridium Escherichia Roseburia Prevotella Desulfovibrio Faecalibacterium Ruminococcus Parabacteroides Blautia Enterococcus Oscillospira Alloprevotella Staphylococcus Lactococcus Adlercreutzia Allobaculum Anaerostipes Eubacterium Alistipes Bacillus Ruminiclostridium Butyricoccus Helicobacter Oscillibacter Anaerovorax Turicibacter Lysinibacillus Barnesiella Turicibacter Delftia Dorea Butyrivibrio Anaeroplasma Tyzzerella Rikenella Romboutsia Coprococcus Anaerotruncus Tannerella Methanobrevibacter Mucispirillum Jeotgalicoccus Pseudomonas Yersinia Streptococcus Corynebacterium Aerococcus Anaerotruncus Mucispirillum Phascolarctobacterium

Number of reports

Increase Decrease

Figure 1. The number of reported changes of intestinal bacterial genera in dietary factor intervention studies in animals.

Roseburia [141] is an enteric bacterium that utilizes dietary fiber and may enhance intestinal barriers by producing butyric acid. It has been reported that administration of Roseburia to mice

enhanced differentiation of regulatory T cells in the intestine and suppressed intestinal

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inflammation [142]. As described in Table 4, oligofructose, apple-derived polymeric procyanidins, sea cucumber-derived sulfated polysaccharide, camu camu extract, and ganoderma lucidum mycelium

water extract were reported to increase the proportion of Roseburia in the gut flora.

Roseburia metabolizes oligofructose into fructose, which is used for growth, but for this process, acetic acid that is produced by Bifidobacterium is required [143]. Therefore, in order to grow Roseburia by oligofructose intake, it is necessary to pay attention to the symbiotic relationship with other intestinal bacteria and the amount of short-chain fatty acids in the intestine. Other dietary factors, procyanidins, sea cucumber-derived sulfated polysaccharide, camu camu extract, and ganoderma lucidum mycelium, have not been studied for their prebiotic function for Roseburia.

It has been suggested that LPS from Prevotella has fewer phosphate and acyl moieties contributing to endotoxin activity, resulting in a lower TLR4 stimulatory capacity than LPS from Salmonella [144]. Therefore, by increasing the population of Prevotella in the intestinal flora, endotoxin activity in the intestinal contents and damage to intestinal epithelial cells

might be decreased, leading to the reduction of blood LPS levels.

On the other hand, *Prevotella* produces succinate as a metabolite of sugar metabolism [145]. It has also been reported that succinate from intestinal bacteria is utilized by and promotes growth of *Salmonella* serovar Typhimurium [146] and *Clostridium difficile* [147], which are the pathogens of pseudomembranous colitis. Succinate has also been reported to induce colitis via succinate receptors and to promote colonic fibrosis [148]. In addition, proportion of *Prevotella* in the gut flora has been reported to be positively correlated with blood LPS levels in patients with type 2 diabetes [149]. Thus, an increase in the proportion of *Prevotella* does not necessarily have a positive effect on intestinal health. It is necessary to carefully investigate the contribution of *Prevotella* to blood LPS levels. *Clostridium*, *Escherichia*, and *Desulfovibrio* are bacterial genera that may contribute to the increase of blood LPS levels. Many pathogenic bacteria (such as enterohemorrhagic *Escherichia coli*, *Clostridium*

*botulinum*, *Clostridium tetani*, and *Clostridium perfringens*), which produce effector proteins or enterotoxins that disrupt epithelial tight junction belong to these genera [150]. In addition, the endotoxin activity of LPS in non-pathogenic *Escherichia* is also higher than in *Bacteroides*, and an increased proportion of these *Escherichia* in enteric flora aggravate colitis [151]. *Clostridium* species catabolize cholic acid to deoxycholic acid for their growth [152].

It is reported that, in mice, deoxycholic acid increases intestinal permeability through the reduction of goblet cell number, suppression of mucin production, induction of low-grade inflammation, and suppression of tight junction protein (ZO-1) expression [153]. In terms of dietary factors that reduce *Escherichia*, there are many reports of sulfated polysaccharides (Table 4). We could not find any reports that suggested the direct inhibitory effect of sulfated polysaccharide on growth of *Escherichia*. On the other hand, it is suggested that *Bacteroides*, that can be preferentially grown in sulfated polysaccharide feeding, compete with *Escherichia* in the co-culture assay [127]. In order to elucidate the mechanism by which sulfated polysaccharides reduce the proportion of *Escherichia*, it is hoped to study focusing on the interaction between gut microbes. Among the dietary factors that reduce *Clostridium*, procyanidin is reported to decrease the growth of *Clostridium* in fecal batch culture [154]. The bactericidal activity of methanol extract of nopal against *Clostridium* has also been reported [155]. *Desulfovibrio* is a gram-negative, obligate anaerobe, sulfate-reducing bacterium. *Desulfovibrio* utilizes electrons supplied by the oxidation of lactic acid in the electron transport system of the respiratory chain, uses sulfuric acid as the final electron acceptor, and produces hydrogen sulfide as a metabolite [156]. *Desulfovibrio* is ubiquitous in the intestines of humans and mice. Of the studies that showed significant changes in the proportion of *Desulfovibrio*, most studies reported that the proportion was increased associated to the reduction of blood LPS levels (Table 4).

However, it is also

reported that proportions of *Desulfovibrio* increased in the colon of patients with ulcerative colitis [157] and has attracted attention as a pathogen of colitis. In addition, Xie et al. reported in mice that the increase of *Desulfovibrio* in feces was positively correlated with the increase of LPS levels in feces, liver, and blood [45]. Qui et al. reported that ingestion of a high-fat diet in mice increased fecal

Clostridium and Desulfovibrio, and oral administration of these bacteria to the normal chow-fed mice increased fecal and blood LPS levels [158]. These reports suggest that Desulfovibrio plays an important role as a source of LPS in the intestine. Desulfovibrio also competes with Anaerostipes caccae for lactic acid produced by Bifidobacterium, and reduces butyric acid production by inhibiting the growth of Anaerostipes caccae [159]. In addition, as the coexistence of Desulfovibrio and Bifidobacterium inhibits the growth of Bifidobacterium [159], this suggests that the amount of acetic acid produced by Bifidobacterium might be also reduced. On the other hand, it has also been reported that oral administration of Desulfovibrio increases the amount of hydrogen sulfide in the intestinal tract and inhibits intestinal peristalsis [160]. Desulfovibrio is thought to play an important limiting role in increasing blood LPS

levels by supplying LPS, decreasing intestinal barrier function due to reduction of short-chain fatty acid content, and prolonging retention time of intestinal contents due to inhibition of peristalsis (Figure 2). However, despite Desulfovibrio being an important target for metabolic endotoxemia, few dietary factors have been reported to reduce the proportion of Desulfovibrio (Tables 1–4).

a metabolite [156]. Desulfovibrio is ubiquitous in the intestines of humans and mice. Of the studies that showed significant changes in the proportion of Desulfovibrio, most studies reported that the proportion was increased associated to the reduction of blood LPS levels (Table 4). However, it is also reported that proportions of Desulfovibrio increased in the colons of patients with ulcerative colitis [157] and has attracted attention as a pathogen of colitis. In addition, Xie et al. reported in mice that the increase of Desulfovibrio in feces was positively correlated with the increase of LPS levels in feces, liver, and blood [45]. Qui et al. reported that ingestion of a high-fat diet in mice increased fecal Clostridium and Desulfovibrio, and oral administration of these bacteria to the normal chow-fed mice increased fecal and blood LPS levels [158]. These reports suggest that Desulfovibrio plays an important role as a source of LPS in the intestine. Desulfovibrio also competes with Anaerostipes caccae for lactic acid produced by Bifidobacterium, and reduces butyric acid production by inhibiting the growth of Anaerostipes caccae [159]. In addition, as the coexistence of Desulfovibrio and Bifidobacterium inhibits the growth of Bifidobacterium [159], this suggests that the amount of acetic acid produced by Bifidobacterium might be also reduced. On the other hand, it has also been reported that oral administration of Desulfovibrio increases the amount of hydrogen sulfide in the intestinal tract and inhibits intestinal peristalsis [160]. Desulfovibrio is thought to play an important limiting role in increasing blood LPS levels by supplying LPS, decreasing intestinal barrier function due to reduction of short-chain fatty acid content, and prolonging retention time of intestinal contents due to inhibition of peristalsis (Figure 2). However, despite Desulfovibrio being an important target for metabolic endotoxemia, few dietary factors have been reported to reduce the proportion of Desulfovibrio (Tables 1–4).

Figure 2. A hypothetical schematic of the behavior of Desulfovibrio in the intestine, influx of LPS, and the effects of sulforaphane on Desulfovibrio. Desulfovibrio, a source of LPS, reduces the amount of shortchain fatty acids in the intestinal tract through lactic acid consumption and suppression of growth of Bifidobacterium, thereby attenuating tight junction. In addition, hydrogen sulfide, a metabolite of Figure 2. A hypothetical schematic of the behavior of Desulfovibrio in the intestine, influx of LPS, and the effects of sulforaphane

on *Desulfovibrio*. *Desulfovibrio*, a source of LPS, reduces the amount of short-chain fatty acids in the intestinal tract through lactic acid consumption and suppression of growth of *Bifidobacterium*, thereby attenuating tight junction. In addition, hydrogen sulfide, a metabolite of *Desulfovibrio*, inhibits peristalsis, thereby retaining the LPS-containing intestinal contents and promoting LPS absorption. The functional component of the broccoli sprouts, glucoraphanin, is metabolized by enteric bacteria to sulforaphane. Sulforaphane inhibits the growth of *Desulfovibrio* and the entry of LPS into the blood (details are described in the Section 4). LPS: Lipopolysaccharide, GR: Glucoraphanin, SFN: Sulforaphane. Broccoli and sprouts illustrations © irasutoya, 2012 and 2013, respectively. Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane) is an isothiocyanate with an N=C=S functional group and is abundant in broccoli (especially the sprout) and other cruciferous vegetables as the precursor glucoraphanin. Sulforaphane is thought to play a role in plant protection through its antimicrobial action [161], induction of programmed cell death of infected tissue [162], and inhibition of insect feeding [163]. On the other hand, in humans and rodents, sulforaphane activates

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NF-E2-related factor 2 (NRF2), which induces expression of genes expressing antioxidant and detoxication enzymes, including phase II enzymes, and then exerts anti-cancer [164], anti-liver damage [165], and anti-depressive effects [166]. We found that dietary administration of broccoli sprout extract reduced blood LPS levels and attenuated obesity, glucose intolerance, hepatic steatosis, and inflammation in mice fed a high-fat diet [94]. We also reported that the proportion of *Desulfovibrionaceae* [upper taxa (family) of *Desulfovibrionaceae*] was positively correlated with blood LPS levels, and that ingestion of broccoli sprout extract reduced *Desulfovibrionaceae* in cecal contents. Subsequently, Wu et al. also reported that broccoli powder reduced the proportion of *Desulfovibrio* in the large intestinal contents of mice [167].

They reported that

the decrease in *Desulfovibrio* composition was negatively correlated with the activity of myrosinase-like activity, isothiocyanate content, and NAD(P)H:quinone dehydrogenase 1 (NQO1) in the colonic mucosa. Ingested glucoraphanin is metabolized by myrosinase-like enzymes in enteric bacteria, which then produce sulforaphane [168]. Since sulforaphane enhances NQO1 activity through activation of NRF2 [168], it is suggested that sulforaphane metabolized and formed from glucoraphanin in broccoli sprouts may have an inhibitory effect on *Desulfovibrio* (Figure 2). Sulforaphane has been reported to exert antibacterial activity against the Proteobacteria (*Desulfovibrio* belongs to this phyla) [169], but its direct effect on *Desulfovibrio* is not well understood. It is hoped that the mechanism by which sulforaphane decreases the proportion of *Desulfovibrio* will be elucidated.

5. Conclusions In this article, we summarized previous reports about the regulation of metabolic endotoxemia through dietary factors, focusing on gut microbiota.

Although changes in the composition of Firmicutes and Bacteroides due to excessive fat intake have been reported to contribute to metabolic endotoxemia in many reports, the results differ between studies and between species, and further investigation is needed to find true pathobionts. Moreover, since human epidemiological studies have not found a correlation between fat intake and blood LPS levels, it is necessary to search for dietary factors other than fat that cause metabolic endotoxemia. Regarding dietary factors that improve metabolic endotoxemia, human intervention studies have focused on probiotics, prebiotics,

polyphenols and dietary habits, and it has been reported that prebiotics, including oligosaccharides, are effective. On the other hand, few studies have evaluated the effects of dietary intervention on gut flora in humans. The development and popularization of next-generation sequencing has made it possible to comprehensively analyze the “fecal” microbiota in humans. On the other hand, as mentioned above, there are also mucin-adherent bacteria that are thought to be involved in metabolic endotoxemia (e.g., *Akkermansia* and *Bacteroides*). In a colitis mouse model, it has been reported that the bacterial flora in the mucin layer exhibits changes from 12 weeks before the onset of colitis, and that the mucin layer was thinned [170]. In this study, changes in the fecal flora occurred at the same time as the onset of colitis, indicating that the bacteria in the mucin layer play an important role in understanding the physiological state of the intestinal tract. However, although it is possible to collect mucin layer samples in animals, it is not easy to do so in humans, due to ethical and technical obstacles. In the future, if a method for collecting the mucin layer in a noninvasive manner is established in humans, the research field of metabolic endotoxemia can be further advanced. Then, it is expected that we will comprehensively understand the relationship between dietary factors, dysbiosis, and metabolic endotoxemia in humans by conducting human intervention studies and epidemiological studies with dietary surveys, gut microbiota analysis using next-generation sequencers and evaluation of blood LPS levels.



physiology, have been attributed to gut microbiota, including stimulation of the immune system, control of pathogenic bacterial proliferation, production of short-chain fatty acids, and fermentation of amino acids and saccharides [1,2]. A disequilibrium of the intestinal microbiota is called dysbiosis, and it causes an alteration of the intercellular tight junctions, allowing access of pathogens (and their toxins, in particular bacterial

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lipopolysaccharides) and stimulation of the mucosa-associated lymphatic tissue (MALT) with activation of the inflammatory cascade (leukocytes, cytokines, TNF- $\alpha$ ), establishment of a chronic inflammation process (Figure 1) and, consequently, tissue damage [1].

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Figure 1. The delicate equilibrium between eubiosis and dysbiosis in the bowels. Eubiosis is the condition in which saprophytic bacteria are present in the mucus-microbiotic layer of the bowel (either the small or the large one). Dysbiosis is a condition in which pathogenic bacteria (Pathogenic bacteria are represented with purple frame, non-pathogenic have a blue frame) predominate and cause changes in the intercellular tight junctions and, in turn, activation of the MALT, leading to tissue damage.

For this reason, dysbiosis has been implicated in the onset of several chronic autoimmune or inflammatory pathologies, including IBD (e.g., ulcerative colitis (UC) and Crohn's disease (CD)), metabolic diseases (e.g., obesity, type diabetes 2 and nonalcoholic fatty liver disease), autoimmune diseases (e.g., rheumatoid arthritis, allergies, and systemic lupus erythematosus), and other disorders (e.g., food intolerances and even colorectal cancer) [1,3]; to date, only few studies linked dysbiosis to primary or secondary immunological oral mucosal disorders [4–6]. The onset of extra-intestinal pathologies linked with dysbiosis is due to bacterial signals that affect the innate and adaptive immune system. These signals also involve type 3 innate lymphoid cells, which contribute to the differentiation of T and B cells and induce the production of Th17 cells through secretion of IL-22. Moreover, it has been shown that the intestinal microbiota influences the accumulation of IgA-producing cells in the lumen, and that IgA diversity in the intestine is related to changes in microbiota composition. The intestinal microbiota also promotes the differentiation of naive CD4+ T cells into Th17, which act at the epithelial level to improve the integrity of the intestinal mucosal barrier. This suggests that a disruption at this level can cause changes in the intestinal barrier and the onset of various pathologies [7] affecting not only the bowel (e.g., UC), but also other organs (i.e., arthritis, uveitis, etc.).

1.1. Oral Manifestations in IBD Apart from the main symptoms related to the gastrointestinal involvement typical of IBD, these patients may present a broad spectrum of non-intestinal signs and symptoms known as extraintestinal manifestations (EIMs): joints, skin, eyes, the biliary tract and the oral mucosa are the most common sites involved [8].

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It is estimated that approximately one third of IBD patients may develop EIMs [9]; in particular, oral lesions may anticipate or accompany gastrointestinal illness: patients with IBD may present these manifestations years before the appearance of intestinal symptoms (5–10%), but EIMs are most commonly diagnosed after intestinal involvement has occurred [10]. EIMs can sometimes be confused with other extraintestinal complications related to IBD due to malnutrition, chronic inflammation or side effects of drugs used to treat the disease itself [11]. The etiopathogenesis, classification, and natural history of muco-cutaneous disorders related to IBD have not yet been well defined: in general, oral lesions are found more often in patients with CD compared to UC, in children compared to adults and in males compared to females [8]. Differences may be found in the presentation of oral lesions between CD and UC: CD is characterized by both specific and nonspecific oral lesions, while only nonspecific ones are found in UC. In CD, oral lesions are defined as specific if the histopathologic data shows evidence of granulomas (similar to those observed endoscopically in the intestine); these are less common than nonspecific lesions (shown in Table 1) and they include: indurated tag-like lesions, cobblestoning, mucogingivitis, lip swelling, deep linear ulcerations and midline lip fissuring (Table 2).

Table 1. Clinical characteristics of nonspecific oral lesions in CD.

Nonspecific Oral Lesions Clinical Presentation Aphthous stomatitis Shallow, round ulcers surrounded by an erythematous halo with a central fibrin membrane Angular cheilitis Erythema with/without painful fissures and sores at the corners of the mouth Glossitis Painful atrophy of the tongue

Pyostomatitis vegetans

Small exophytic lesions covered with a vulnerable membrane, their cracking and confluence results in the characteristic sign of a “snail track” Oral Lichen/Oral Lichenoid reactions Associated to taste disturbances Gingivitis/Periodontitis Associated to a vitamin D deficiency

Table 2. Clinical characteristics of specific oral lesions in CD.

Specific Oral Lesions Clinical Presentation Indurated tag-like lesions (mucosal tags) White reticular tags (labial and buccal vestibules, retromolar region) Cobblestoning Fissured and corrugated swollen mucosa with hyperplastic appearance (posterior buccal mucosa) Mucogingivitis Edematous, hyperplastic and granular gingiva (whole gingiva up to the mucogingival line) Lip swelling Associated to vertical fissures Deep linear ulcerations Associated to hyperplastic margins (vestibule) Tongue and midline lip fissuring Lip and tongue fissures

In particular, cobblestoning and tag-like lesions are considered pathognomonic for CD, but these are generally not associated with active intestinal disease [8]. Nonspecific oral lesions are found more often than the specific ones, and are usually associated with CD and UC; these include recurrent aphthae, angular cheilitis, pustular ulcerations, pyostomatitis vegetans, glossitis, lichen planus and nonspecific gingivitis [12]. Among the nonspecific findings, recurrent aphthae are the most known oral lesions associated with IBD; when the onset of aphthae is associated with systemic disorders, the term ALU is now

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considered preferable over the previously used RAS (recurrent aphthous stomatitis), since aphthae are considered a secondary manifestation and the different clinical courses of the two conditions require different management strategies [13]. ALU are reported to occur in up to 10% of UC and 25% of CD patients, and they may become more severe in active disease; however, their presence or absence does not correlate with disease activity [14].

Clinically, ALU are shallow, round or oval shaped lesions, granular on palpation; they are often painful, leading to negative effects on patients' daily activities [15]. Their onset is usually sudden and may be concurrent with a flare-

up of intestinal symptoms, or appear simultaneously with other EIMs [16]. Data from literature are conflicting in terms of the association between ALU and the pathological activity: some studies link the presence of oral lesions with the concomitant presence of intestinal symptoms, while other contradicting findings report no statistically significant difference [17,18]. Deficiencies caused by diet or poor absorption of an essential nutrient can cause anaemia and

mineral and vitamin deficiency; in particular, vitamins B1, B2, B6, B12, iron, serum ferritin and folic acid deficiencies have been reported in the pathogenesis of oral ulcers, implicating their role in weakening the immune system [19]. Anaemia may also arise from chronic intestinal bleeding associated with iron deficiency, causing angular cheilitis and painful depapillation of the tongue; constant iron and zinc deficiencies may also be linked to erosive and crusty lesions on the lip commissures and perioral region [20].

Regarding IBD therapy, all of the currently used drug classes have been linked to alterations in the

oral cavity due to their direct toxic effect on tissues and their indirect immunosuppressive effects [21].

1.2. Morphology: Comparative Microscopic Anatomy of Oral and Intestinal Mucosa All the organs of the alimentary canal have a common origin in the primitive digestive gut. The oral mucosa, that covers the entire oral cavity, consists of two layers: a stratified squamous epithelium and an underlying connective tissue (lamina propria) that includes blood and lymphatic vessels, as well as nerves and immune cells. However, the oral mucosa varies in structure, function and appearance in different regions of the cavity, and it is divided into lining, masticatory and specialized (gustative) mucosa [22,23], as detailed in Figure 2. Both the lining and masticatory oral mucosae may host aphthous lesions.

The wall of the small and large bowel is canonically divided into four layers: mucosa, submucosa, muscularis propria and serosa or adventitia. The mucosa is composed of epithelium, lamina propria and muscularis mucosae. A simple columnar epithelium covers the small and large bowel (Figure 2), although many regional differences are present [24–26]. The similarities between the oral and the intestinal mucosa include: (1) the presence of tight junctions between epithelial cells; and (2) the presence of a basement membrane between the epithelium and the lamina propria. The equilibrium between the epithelium and the lamina propria is very important for mucosal homeostasis, and both alterations in tight junctions and changes in basement membrane may result in dysfunction of the mucosal barrier, as seen in inflammatory bowel disease [27].

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Figure 2 Comparison between oral (both lining and masticatory, respectively subfigure a and b) and bowel (both small and large, respectively subfigure c and d) mucosae. Above: Original pictures, hematoxylin and eosin stainings; bar: 100 micra. Original magnifications: 100×. Below: drawings summarizing the main characteristics of these tissues. In detail, the epithelium of oral mucosa is a stratified squamous epithelium, non-keratinized in the lining mucosa (subfigure a) and keratinized in the masticatory mucosa (subfigure b). It is divided into four layers: basal layer, prickle-cell layer, intermediate layer and superficial layer for lining mucosa; and basal layer, prickle-cell layer, granular layer and superficial (keratinizing) layer for masticatory mucosa. In both epithelia, the basal layer consists of cuboidal or columnar keratinocytes that are capable of division so as to maintain a constant

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layer, prickle-cell layer, granular layer and superficial (keratinizing) layer for masticatory mucosa. In both epithelia, the basal layer consists of cuboidal or columnar keratinocytes that are capable of division so as to maintain a constant epithelial population. Cells arising by division in the basal layers of the epithelium undergo a process of maturation as they are passively displaced toward the surface. In the non-keratinized squamous epithelium, the cytoplasm of intermediate cells does not contain keratin filaments. In keratinizing epithelium, the granulosum stratum is prominent and cells contain intracytoplasmic granules of keratohyaline. The epithelium of small bowel (c) covers the intestinal villi and the crypt compartments; it is columnar and composed of various cell types, such as absorptive cells, goblet cells and endocrine cells, in the villi, and stem cells and Paneth's cells, in the crypts. The epithelium of large bowel (d) covers glandular crypts; it is composed of a single layer of columnar cells and consist of absorptive cells that are responsible of water and ion transport, and goblet cells.

1.3. The Intestinal Microbiota and the Surrounding Mucous: The Fifth Layer of the Bowel Wall As stated before, the wall of the bowel is canonically divided into four layers by morphologists

through the observation of histological sections after processing them with reagents, including alcohols that remove mucus and other alcohol-soluble substances. However, in living subjects, the mucosal layer is characterized by the presence of a mix of symbiotic and pathogenic bacteria embedded in the mucus, produced by the epithelial cells. In this mucous matrix, apart from bacteria, are present a number of soluble substances and nanovesicles (i.e., exosomes, microvesicles and outer membrane vesicles), produced by both human cells and bacteria, that actively participate in the regulation of the homeostasis of the intestinal mucosa and, consequently, through lymphatic and hematic circulation, of virtually all of the organs [28–30]. Therefore, as already proposed [22], this mucus-microbiotic layer can be considered the real innermost layer of the intestinal wall.

The relevance of this hypothesis lies in the

fact that the understanding of the pathogenesis of human diseases derives from a precise knowledge of

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normal morphology, since many (if not all) pathologies derive from an alteration in cell differentiation that, in turn, generates tissutal changes and loss of organ function; thus most (if not all) treatment strategies should aim—when possible—to restore the normal morphology of the organs. The aim of the present paper is to present a novel pathobiological hypothesis and, consequently, a non-invasive therapeutic method.

2. Pathobiology of ALU in IBD and Therapeutic Proposal

2.1. Pathobiology: A Focus on Dysbiosis The persistence of dysbiosis causes a state of chronic inflammation linked to the activation of MALT and the release of inflammation mediators. This causes an onset of pathologies even in areas that are physically far away from the gut [1–3].

Intestinal epithelial cells represent the main communication barrier between the host environment and the microbiota, and also regulate the impact of the microbiota on the host immune function (Figure 3). For example, a healthy intestinal microbial flora promotes regulatory B (Breg) cell differentiation and IL-1b and IL-6 production, and controls inflammatory processes through Breg cells and IL-10 secretion.

Gut microbiota influences not only local, but also systemic immunity by bacterial metabolites

(such as ligands of aryl hydrocarbon receptor and polyamines) and bacterial components, such as polysaccharide A, that exhibit immunomodulatory action [31]; moreover, studies carried out on animal models have shown that intestinal dysbiosis may be related to the development of autoimmune diseases. Int. J. Mol. Sci. 2018, 19, x FOR PEER REVIEW 6 of 12

Figure 3. Aphthous-like ulcers: histopathological features. Left: These images show some typical features of ALU: round or oval ulcer covered by a yellow-white fibromembrane with a peripheral erythematous halo; margins may appear indurated and elevated (A: ALU in the right side of the tongue; B: ALU in the posterior buccal mucosa; C: Four concomitant ALU in the anterior buccal mucosa). Right: The ulcerative lesion shows an increased angiogenesis and a mixed inflammatory infiltrate that consists of various leukocytes (lymphocytes, neutrophils, monocytes and histiocytes).

Mechanisms with which intestinal dysbiosis could generate autoimmune activation are not yet well understood. It is thought that they may be related to: - Alteration of Treg/Th17 due to dysregulated TLRs on antigen-presenting cells [27]; - Resistance to colonization, i.e., ability of the gut microbiota to limit the proliferation of external pathogens. It has been observed that in patients with autoimmune disorders (e.g., systemic lupus erythematosus), resistance to colonization was lower than in healthy controls; - Superantigens, derived from bacteria and viruses that have the ability to activate immune cells by simultaneously binding to the major proteins of the class II histocompatibility

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Right:

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creation of neoantigens [27,33]; - Mucosal responses to microbiota, i.e., inflammatory cytokines that activate nearby autoreactive cells [30]; - Molecular mimicry, i.e., cross-reactive antibody that recognizes shared epitopes of microbial and host tissue proteins, and activation of autoreactive T and B cells [29,34,35].

Thus, the hypothesis we formulate is that ALU is the result of the concomitance of intestinal dysbiosis (and consequent activation of the immune system) and other events, e.g., the microtraumas occurring (frequently and for various causes) in the oral mucosa.

Microtraumas can be considered as a stress factor for oral mucosa that induce overexpression, trafficking and surface mislocalization of intracellular proteins that may work, pathogenetically, as autoantigens. Heat shock proteins are an example of intracellular proteins that—after cell stress—may be mislocalized to cell surface by post-translational modifications that trigger unusual intracellular trafficking pathways; in addition, bacterial Hsp60 homologous, i.e., GroEL, can induce the formation of antibodies against it that can also cross-react against surface-exposed Hsp60, generating an autoimmune response by a molecular mimicry mechanism [36–39].

2.2. ALU Treatment: Can Probiotics Be Useful? In view of the above hypothesis, we suggest that an ex adjuvantibus therapy with probiotics could be able to modify the natural course of ALU (Figure 4).

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Figure 4. Probiotics as effective therapy for ALU. Restoration of eubiosis can dramatically contribute to remission of ALU by contrasting pathogenic phenomena. (Pathogenic bacteria are represented with purple frame, non-pathogenic with a blue frame; the yellow and green frame indicate the bacterial strains present after administration of probiotics)

We cannot yet precisely answer the question “how do probiotics work?” but some theories can be formulated. There are strong functional similarities between the gut and oral biofilms: it is reasonable to speculate that corresponding health-promoting events may occur in the oral cavity to those already reported in the gut. The oral cavity is a large reservoir of bacteria of >700 species and it is closely related to host health and disease [40,41]. In a recent study, it is demonstrated an association

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Probiotics as effective therapy for ALU. Restoration of eubiosis can dramatically contribute to remission of ALU by contrasting pathogenic phenomena. (Pathogenic bacteria are represented with purple frame, non-pathogenic with a blue frame; the yellow and green frame indicate the bacterial strains present after administration of probiotics).

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occur in the oral cavity to those already reported in the gut. The oral cavity is a large reservoir of bacteria of >700 species and it is closely related to host health and disease [40,41]. In a recent study, it is demonstrated an association between dysbiosis of the salivary microbiota and IBD patients; it was observed that the salivary microbiota in IBD patients significantly differed from that of healthy ones, and found particular bacterial species associated with dysbiosis (*Prevotella* and *Veillonella* were significantly higher in both the CD and UC groups while *Streptococcus*, *Haemophilus*, *Neisseria* and *Gemella* were significantly lower compared with the healthy ones). It was also showed that the dysbiosis is strongly associated with elevated inflammatory response of several cytokines with depleted lysozyme in the saliva of IBD patients [42].

In the oral cavity, probiotics create a biofilm which matches with carcinogenic bacteria and periodontal pathogens modulating host immune response by strengthening the immune system [43]. There are local (direct) as well as systemic (indirect) events that occur by regulation of the immune response. The potential pathways could include [44]:

- Co-aggregation and growth inhibition;
- Bacteriocin and hydrogen peroxide production;
- Competitive exclusion through antagonistic activities on adhesion and nutrition;
- Immunomodulation.

There is an increasing body of evidence suggesting that perturbations of mucosal microbiota can modulate innate and adaptive immune responses, with inflammation arising upon reduction of the number of symbiont microorganisms and/or increase in the number of pathobiont microorganisms (commensal bacteria with pathogenic potential) [45]. Several immune mechanisms, implicated in the remission of ALU, by symbiont bacteria have been hypothesized, including induction of IL-10, suppression of TNF- $\alpha$  and IL-8, and modulation of Toll-like receptors [46].

This hypothesis has been reinforced by some studies that correlate the administration of probiotics to the improvement of autoimmune diseases. For example, it has been observed that in patients with rheumatoid arthritis, the administration of *Lactobacillus casei* increased the serum levels of IL-10 anti-inflammatory cytokine and decreased the levels of proinflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-12 [27].

3. Conclusions Intestinal dysbiosis causes a chronic inflammatory state and activation of the MALT in the gut, which leads to the onset of extraintestinal pathologies [1–3]. We hypothesized that ALU could also be caused by intestinal dysbiosis, due to the immunological mechanisms involved in the pathogenesis of the disease [27] and the fact that there are several immune mechanisms implicated in the remission of ALU mediated by symbiont bacteria [36]. By comparing what happens in the intestine [47], we hypothesize that the administration of probiotics can increase the expression of tight junction protein ZO-1, both in terms of transcriptome and protein synthesis, with an improve intestinal barrier function. In fact, it was shown as a result of a chronic inflammatory state levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-23 stimulate the epithelial barrier breakdown, affecting in particular the expression of proteins forming the tight junctions.

This increase can be countered by administering specific probiotic strains including *Lactobacillus salivarius*, *Bifidobacterium lactis*, *Lactobacillus Plantarum* and *Lactobacillus fermentum* [48,49]. In addition to antagonistic act on proinflammatory cytokines, microbial metabolites directly promote the synthesis of the aforementioned tight junction proteins through activation of aryl hydrocarbon receptor, with subsequent activation of nuclear

factor erythroid 2-related factor 2 (Nrf2) which has as a final result just the increased synthesis of ZO-1 with consequent strengthening of the epithelial barrier [50].

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Therefore, we proposed the use of probiotics as direct therapy for intestinal dysbiosis and as an indirect one for ALU. In particular, preliminary observations lead us to suggest that the therapy with probiotics should be started when the patient first starts to experience the ALU symptoms. Our hypothesis is that this therapy can reduce the duration of the disease by up to three days, through limiting the development of the lesion and favouring the re-epithelization of the lesioned oral cavity mucosa thanks to the molecular mechanisms discussed above (Figure 5). Int. J. Mol. Sci. 2018, 19, x FOR PEER REVIEW 9 of 12

Figure 5. Probiotics as effective therapy for ALU. Hypothesis: Restoration of eubiosis can dramatically contribute to remission of ALU by contrasting pathogenic phenomena. Experimental and clinical evidence on the use of probiotics for the treatment of oral aphthae are currently very limited, and the etiology and pathogenesis of ALU is currently unknown. So, we think that it would be opportune to carry out in depth studies of this phenomenon, taking into account that host genetics, nutritional deficiencies, and a number of systemic conditions have been recognized as systemic modulating factors of ALU [34,51]. Further studies are needed to establish which immunological mechanisms can be implicated in ALU pathogenesis and modulated by the administration of probiotics.  
Funding:

Cardiovascular diseases (CVD) remain the chief cause of death in both western and developing societies (1). Despite the enormous growth in knowledge and advances in prevention and treatment, approximately one out of three people in the USA still die from CVD (2). In addition to traditional risk factors such as hypercholesterolemia, homocystinemia, hypertension, hyperglycemia, cigarette smoking, and aging, metabolic endotoxemia (ME) has been suggested to contribute to endothelial injury and development of CVD (3, 4). Nowadays attentions have been attracted to the role of ME in many fields of medicine particularly inflammatory diseases like atherosclerosis and other types of CVD (5). In ME, microbiome-derived lipopolysaccharide (LPS) from the gut microbiota passes through the intestinal mucosa to enter the bloodstream, and may represent an important mediator of low-grade systemic inflammation (6). Previous studies especially in patients with chronic kidney disease (CKD) have shown that high levels of endotoxin lead to production of pro-inflammatory cytokines and may predispose these patients to CVD (7). More recently, increased level of Trimethylamine-N-oxide (TMAO), a gut bacterial metabolite, has been suggested as a new risk factor in CVD development. Changes in gut microbiota (dysbiosis) seem to contribute to ME. Under normal conditions, the intestinal epithelium acts as an impervious barrier to prevent LPS translocation; however, some conditions may alter this protective function (8). Dysbiosis is defined as “any change to the components of resident commensal community relative to the community found in healthy individuals”. Each of the following three conditions are generally classified as dysbiosis: 1) loss of valuable microbial organisms, 2) expression of pathobionts of possibly beneficial microorganisms, 3) loss of general microbial variety (9). In states of dysbiosis, the intestinal barrier increases in permeability as a result of a

disruption to the regulation of the epithelial cell-to-cell tight junction protein network (10). A compromised intestinal barrier can be associated with bacterial translocation from the gut into the systemic circulation increasing the risk of ME (11, 12). Disruption of the gut barrier and translocation of LPS and other bacterial metabolites have been shown to affect many aspects of human health, through various gut-to-organ axes; some examples include gut-brain axis, gut-heart axis, gut-skin axis, etc. The interaction between the gut and a specific organ has received much attention in current years. Although gut microbiota imbalance has been postulated to be associated with CVD through endotoxemia, it is yet to be explored whether dysbiosis leads to inflammatory-mediated CVD risk, or CVD dysregulates gut microbiota composition by impairing blood supply of the gut (13).

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In the current review, we will debate findings on probable mechanisms connecting the gut microbiota and onset of endotoxemia. Additionally, we will discuss the potential relationship between ME and CVD. Finally, we will review the evidence on the potential role of prebiotics/probiotics in modulation of gut microbiota and host metabolism with regard to the development of ME.

**RESULTS** Selected articles A flow diagram of the study selection is summarized in Fig 1. In total, 6895 articles were retrieved, of which 2560 were duplicates, resulting in 4335 non-duplicated publications. Of these 4335 publications, 4131 articles did not meet the inclusion criteria and were excluded. A further 22 articles were excluded due to insufficient information. After exclusion, 19 articles met the eligibility criteria and were included in this review.

#### Study characteristics

Characteristics and the main outcomes of the 19 articles included in the current review are summarized in Table 1. The studies were conducted between 2007 and 2019. Of all the identified studies, three studies were conducted in animal and 16 studies used a randomized clinical trial design. The trials group ranged in duration from 3 to 28 weeks

**Gut Microbiota** The gut microbiota (formerly called gut flora) is the complex community of microorganisms including bacteria, archaea and eukaryotes that live in the digestive tract of humans (8). The majority of the GI-tract bacterial composition represent only two bacterial phyla, the Firmicutes and the Bacteroidetes. The gut microbiota offers many profits to the host, through a range of physiological functions such as strengthening gut integrity or affecting the intestinal epithelium, harvesting energy, protecting against pathogens and regulating host immunity (14, 15). However, there is a potential for these mechanisms to be disrupted as a result of altered microbial composition, known as dysbiosis (16). Many factors can modify the balance of gut microbiota and allow for translocation of luminal contents to the inner layer of intestinal wall (15). The normal gut

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barrier, supported by tight junctions, prevents translocation of whole bacteria or bacterial fragments/products into the sub-mucosal compartment (14). In the 'leaky gut' situation, infiltration of bacteria or related components into sub-mucosal space results in stimulation of mast cells and lymphocytes. The activation of these immune cells leads to production of proinflammatory cytokines, which further induces chronic inflammation and ME (17).

Balanced gut microbiota plays a critical role in maintaining immune and metabolic homeostasis and protecting against pathogens. However, numerous studies have demonstrated that gut microbiota alteration (dysbiosis) can lead to increased cardiometabolic risk factors such as hypertension, elevated cholesterol, and insulin resistances, which greatly increase the risk of CVD (8, 18). Numerous mechanisms have been proposed to be involved in the role that gut microbiota alterations play in the etiology of CVD; stimulation of immune system, short chain fatty acid production, chronic low-grade inflammation, lipoprotein and bile acid metabolism, and altered endocannabinoid receptor system tone are among these mechanisms (19). More recently, more attention has been focused on the effect of metabolic endotoxemia (ME) in the etiology of CVD (20).

#### Metabolic Endotoxemia

Two to three-fold increase in circulating LPS levels is termed 'metabolic endotoxemia' (21). Components from gut microbiota, such as LPS, lipoteichoic acid, peptidoglycan, flagellin and bacterial DNA, can cause immune system activation. An animal model showed that modest rises (~1.5 fold) in endotoxin level or injection of 300 mg/kg/day of LPS could lead to increased fat deposition, insulin resistance, and chronic inflammation (22). A recent study has demonstrated that systemic LPS administration led to damages in heart mitochondrial DNA and protein by oxidative stress. They revealed that LPS upregulated endothelial cell adhesion molecules, and LPS associated favorably with the pro-atherogenic fraction (23). Although endotoxemia is not necessarily equivalent to increasing LPS, many have defined metabolic endotoxemia as "a situation of chronically elevated plasma LPS". In patients with septic shock, the concentration of endotoxin level is often elevated a 1000 folds or higher compared to healthy controls (20). On the contrary, Cani et al. defined "metabolic endotoxemia as "a situation of chronically elevated plasma LPS at levels 10–50 times lower than during septic conditions" (21). However, there are Accepted Article  
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more than 20 assays for detection of endotoxin markers, which can lead to cell damage, and theoretically multiple organ failure (24, 25).

LPS is thought to be a major inducer of inflammatory responses, suggesting a possible association between intestinal LPS and CVD. The gut microbiota is a huge reservoir of this endotoxin. There are 10<sup>12</sup> bacterial cells per gram of luminal content. Therefore, more than 1 g of LPS may be detected in the intestinal lumen. LPS is one of the main components of the external cell wall of gram-negative bacteria. Thus, it is expected that changes in the barrier permeability facilitates translocation of LPS and other endotoxins into the bloodstream, and the following metabolic consequences (26). LPS binds to LPS-binding protein (LBP). The complex LBP-LPS is presented to cluster of differentiation 14 (CD14) on innate immune cells, which is expressed mainly by macrophages, neutrophils, and dendritic cells; this subsequently mediates signal transduction, including nuclear factor kappa B (NF- $\kappa$ B) activation via TLR4, and contributes to the activation of innate and adaptive chronic inflammatory responses (27). In addition, results from animal studies suggest that LPS exposure directly induces oxidation of low-density lipoprotein (28, 29) (see Fig.2) Increased gut permeability and subsequent elevated circulating LPS has been shown in many cardiovascular conditions (30). Previous studies have postulated that CVD is accompanied with both alterations in intestinal barrier, and increased microbial translocation. However, it is not yet clear whether dysbiosis is the cause or effect of CVD. Furthermore, some taxa of oral microbiota have also been detected in human

atherosclerotic plaques. These data are supported by previous studies that had found epidemiological links between periodontal diseases and CVD (31, 32). In other words, periodontal diseases may be associated with CVD.

As mentioned above, gut microbiome alterations observed in some diseases leads to an increase in serum levels of some gut metabolites such as TMAO (33). On the other hand, dysbiosis leads to increased production of TMAO, which may also contribute to the pathogenesis of CVD (34). For the first time, Kallio et al., introduced this metabolite endotoxemia as the consequence of dysbiosis which was assumed to have a role in CVD development (35). Animal and epidemiologic studies have shown that higher levels of TMAO are directly linked to the increased incidence of major adverse cardiovascular events (MACE) (36, 37). Indeed, some studies have demonstrated that increased TMAO levels may better predict incident cardiovascular events than traditional risk factors such as LDL and C-reactive protein (CRP) (38). In fact, the smallest microbiota changes even without disrupting gut permeability, cause metabolic complications and metabolite endotoxemia.

*Helicobacter pylori* offers another example of how the gut microbiota of the host can have a major impact on health (39). Indeed, *H. pylori* is directly or indirectly involved in the development of CVD. Activated release of toxins, pro-inflammatory factors, abnormal lipid metabolism, and altered iron metabolism are the major mechanisms through which *H. pylori* contributes to cardiovascular abnormalities (40). Although, *H. pylori* infection might play a role in increasing the circulating levels of endotoxemia in cardiovascular patients (41), consequently facilitating the onset of CVD, its main effect in development of heart diseases might be through alteration of immune system, resulting in systemic endotoxemia (42).

Small intestine bacterial overgrowth (SIBO), also termed bacterial overgrowth, characterized by the presence of abnormal and excessive numbers of bacteria in the small intestine, has been associated with an increased risk of CVD (43). Although numerous speculations have been suggested regarding the crosstalk between SIBO and atherosclerosis, the exact underlying mechanism remains unclear. Recently, Ponziani et al. provided evidence that SIBO predisposes patients to development of atherosclerosis through reduced matrix Gla-protein (MGP) activation as well as arterial stiffening (44). Furthermore, Oher et al. revealed that SIBO increases endotoxemia via activation of TLR signaling pathway which eventually leads to CVD (45). In short, despite the association between SIBO and CVD revealed in previous studies, no conclusions can be drawn about causality of the association.

In addition to the bacterial components that cause ME, certain bacterial metabolites such as TMAO can also exert negative effects on the circulatory system and increase chronic inflammation. TMAO is a biological compound produced by gut microbiota from dietary phosphatidylcholine, choline, and carnitine (46). Alteration of gut microbiota as identified by increased *Prevotella* and decreased *Bacteriodes* species in gut microbiome leads to higher level of TMAO and susceptibility to CVD (34). In addition, elevated TMAO level is a new prognostic marker in patients with ischemic and non-ischemic cardiomyopathy (47). Moreover, a new study proposed that TMAO may be considered as a biomarker to assess gut barrier permeability (48).

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There is evidence that animals fed with Western diet have greater plasma TMAO concentrations. The augmented levels of TMAO is known to contribute to over expression of pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) and also attenuation of anti-inflammatory cytokines such as IL-10 (37). Moreover, endothelial dysfunction is another pathologic feature that has been related to TMAO. TMAO also alters cholesterol and sterol metabolism, which could act as an important risk factor for CVD (34).

#### Gut Permeability and Metabolic Endotoxemia

The gut epithelium is an efficient barrier that prevents absorption of LPS derived from Gramnegative gut microbiota. Diabetes, high-fat diet, obesity, and CVD are associated with higher gut permeability leading to ME (21). Currently, there are some invasive methods used to detect the gut permeability, which may not be appropriate for clinical purposes. A simple non-invasive method is typically using large molecule oligosaccharide [e.g., lactulose or polyethylene glycols (PEGs) of 1,500 to 4,000 kDa] and low-molecular-weight sugars such as mannitol and L-rhamnose or concentration ratio of lactulose to mannitol (L/M ratio). The sugar molecules such as mannitol are supposed to permeate both transcellularly and paracellularly, so that the ratio of these sugars in plasma or excreted in the urine reflects intestinal permeation (49, 50). It must be noted that small intestine is technically sterile, and use of L/M ratio as an indicator for small intestinal permeability would be misleading, unless SIBO exists. Sucralose has been used instead of lactulose as a measure of whole gut permeability (51).

Another indirect method is to assess the tight junction proteins such as occludin, zonulin-1, claudin-1, claudin-4 in serum which are increased in leaky gut (52). Additionally, LBP has also been used as a gut-blood barrier permeability marker (53). More newly, TMAO has been proposed as a promising biomarker of gut barrier function (48). More recently, plasma levels of citrulline, and also assessment of the inflammatory marker calprotectin in feces have been used as a surrogate marker of small bowel epithelial cell mass (54). Although many techniques exist for evaluation of intestinal permeability, calculating the excretion ratio of lactulose/rhamnose or lactulose/mannitol are more commonly used (55).

Assessments of intestinal permeability are regularly used synonymously with the term “gut barrier function,” while these are not the same. For example, intestinal permeability changes do not essentially reveal changes in antimicrobial production, mucus secretion, or IgA secretion (56). Accepted Article

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Taken together, results of all these tests are influenced by changes in many factors including gastric emptying, intestinal peristalsis, gut blood flow, bacterial degradation, and renal clearance. Therefore, there is no single standard way to evaluate the gut permeability, and it is suggested that a combination of these tests be performed for assessment of intestinal permeability.

#### Key point

Potential pathways of the association between gut dysbiosis and CVD have been demonstrated in various animal and human studies. The intestinal microbiota has a deep influence on mucosa barrier function and the nutritional/metabolic status of its ‘host’ (19). Dysbiosis allows bacterial products such as lipopolysaccharide, or peptidoglycans to enter the circulation (57). Furthermore, the dysbiosis can directly impact the cytokine

production from epithelial cells and innate immune cells (21). These mediators also enter the circulation. LPS itself, and also the inflammatory state it causes may induce the production of oxidized low-density lipoprotein (13, 29). In addition to metabolic endotoxemia, increased TMAO as a gut metabolite may also exert adverse effects on cardiovascular system. TMAO, even in the absence of leaky gut, has been proposed to augment CVD risk. Prebiotics/probiotics could possibly attenuate these adverse effects (33).

## DISCUSSION

### Gut dysbiosis and Cardiovascular disease

Dysbiosis can be implicated in the pathogenesis of CVD through 1) increased LPS (endotoxemia) which can promote the formation of atherosclerotic plaque by acting on TLR4 (57), 2) affecting the metabolism of bile acids (BAs), and the production of TMAO which can impair cholesterol catabolism and induce chronic inflammation (37), and 3) contributing to risk factors such as hypertension and atherosclerosis through chronic inflammation and dyslipidemia (5). In the following sections, we will debate findings on probable mechanisms connecting the endotoxemia and CVDs. Furthermore, we will discuss the evidence on the potential role of prebiotics/probiotics in modulation of gut microbiota and endotoxemia.

### Endotoxemia and Cardiovascular Disease

It is well established that patients with cirrhotic cardiomyopathy have higher LPS levels, and are significantly predisposed to diastolic dysfunction. This finding supports a potential role of ME in Accepted Article

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the aggravation of cardiomyopathy in cirrhotic patients (58). In addition, previous studies have shown a relationship between systemic inflammation and increased CVD (6). However, the potential mechanisms for the observed associations still remain largely unclear. Typically, endotoxemia is present in early CVD and also at the early phases of some diseases (58). Additionally, endotoxemia may activate systemic inflammatory cascade that can not only have an influence on the cardiovascular systems, but also have a distant effect on intestine and its permeability (13). Chronic inflammation following endotoxemia might be a possible mechanism for the association between dysbiosis and CVD (6). Indeed, increased levels of inflammatory markers and LPS have been found in CVD subjects (59). It is, however, uncertain whether increased gut permeability can lead to the development of CVD or whether it is a consequence of a cardiovascular condition (60).

Endotoxemia (without sepsis) is characterized by presence of LPS, the major glycolipid component of the outer membrane of gram-negative bacteria in the blood (8). ME stimulates release of pro-inflammatory cytokines, resulting in systemic inflammation. Components of gram-positive bacteria's cell wall such as lipoteichoic acid or peptidoglycan are recognized by pattern-recognition receptors (PRRs) such as NOD-like receptors and Toll-like receptors (TLRs). TLRs are PRRs that recognize microbe-associated molecular patterns, and include many types, but TLRs2 and TLRs4 are the most important ones. LPS and peptidoglycan (PGN) trigger TLR4 activation, and TLR2 recognizes lipoteichoic acid (LTA) from gram-positive bacteria (61, 62). LPS not only induces endothelial damage, but also increases expression of surface adhesion molecules such as CD14 on inflammatory cells, and stimulates the release of pro-inflammatory cytokines (63). Heightened activation of the immune system in post endotoxemia may predispose the animals to the

development of cardiovascular disease. Epidemiological studies have also shown that ME is associated with CVD (41). However, the role of ME in CVD remains unknown, once not considering the part inflammation plays in this regard; thus, further investigation is warranted.

It has been proposed that ME increases hypertriglyceridemia, and development as well as progression of fatty liver (64). Also, LPS seems to increase endothelial lipase, which has been suggested to cause a reduction in HDL (60). These findings suggest a strong link between ME and increased CVD risk factors. Endotoxins can also induce plaque formation and progression of Accepted Article

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atherosclerotic lesions, and release of other molecules from endothelial cells involved in proinflammatory processes (65).

Several mechanisms have been proposed to be involved in the role of TMAO (considered as metabolite endotoxemia) in the etiology of CVD; activating macrophages to accumulate cholesterol, changing cholesterol metabolism in different organs, and inhibiting reverse cholesterol transport pathway are some of the most important mechanisms (47).

Moreover, elevated TMAO levels promote inflammation and oxidative stress, and impair vascular function (37).

#### Gut Microbiota and Endotoxemia

Dysbiosis may contribute to ME, leading to systematic inflammation, and CVD (5). A healthy intestinal barrier is important to avoid microbial translocation. Evidence from clinical and animal studies show that dysbiosis is associated with an increased risk of CVD (34, 35). Moreover, several lines of evidence suggest that increased gut permeability, as assessed by tight junction proteins in serum, contribute to cardio-metabolic risk factors. Surprisingly, hypercholesterolemia paradoxically improves survival in cardiac cachexia, and attenuates cardiac cachexia and inflammation, suggesting a hypothesis that a diet with high fat content, could decrease gut permeability and subsequently metabolite endotoxemia (66, 67).

As noted before, gut microbiota alterations lead to development of different diseases, such as CVD. Gut microbiota regulates multiple physiological processes of the host; the resident bacteria act as an energy sources in the gut lumen, influence production of leptin and other hormones, regulate immune functions and receptor ligands, and are also substrates for the host enzymes. In order to identify how gut microbiota alterations influence inflammation, high fat diet was used in experimental settings. High fat diet increased plasma endotoxin levels and resulted in dysregulation of the gut microbiota by increasing the ration of Firmicutes to Bacteroidetes. The analyses showed that LPS was responsible for the onset of ME in this animal model (68).

Germ-free animals have been used to study the probable role of the gut microbiota in development of some disorders (69). Germ-free animals are animals that have no microorganisms living in or on them. Such animals are raised within germ-free isolators in order to control their exposure to viral and bacterial agents (70). Germ-free mice fed a normal chow diet had a lower endotoxin production, whereas germ-free mice colonized with LPS-producing germs showed Accepted Article

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increased fat mass, and developed metabolic diseases (71). Earlier investigations have revealed that colonization of germ-free mice with microbiota considerably changes the

transcription of numerous mediators involved in the regulation of metabolic functions (72, 73). Turnbaugh et al. observed that colonization of germ-free mice with the microbiota from the obese mice resulted in a considerably higher percentage of total body fat than that resulting from colonization with a microbiota from lean mice (72). These results elucidate that gut microbiota is another causal factor in pathophysiology of cardiac risk factors. To conclude, endotoxemia and its resultant inflammation is not observed in germ-free mice, but develops only after feeding of high fat diet or injection of LPS to these animals; this in part demonstrates the effect of gut microbiota dysbiosis in this regard.

There is inconsistency regarding the relationship between high fat diet and elevated circulating endotoxin. Pendyala et al. (74) demonstrated that fasting plasma endotoxin was significantly raised following 30 days of isocaloric, high fat (40% fat of total energy) feeding in apparently healthy subjects. On the contrary, 2 months of high fat (45% fat of total energy) diet did not influence fasting plasma LPS in healthy subjects, in another study (75). Apparently, the association between high fat diet and ME is more complex in humans, and seems to be influenced by the time course of feeding, the macronutrient (and possibly energy) composition, and the age of the individuals.

This evidence suggests that changes in gut microbiota composition could be responsible for increased endotoxemia, which in turn would trigger the development of inflammation and cardiovascular risk factors. On the other hand, antibiotic treatment intensely reduces the local intestinal microbiota and LPS. Similar results were observed when a probiotic was administered to mice; Bifidobacteria administration in newborn mice led to lower intestinal endotoxin concentrations and inflammatory cytokine (IL-6, and TNF- $\alpha$ ) production (76).

#### Pre/probiotics and Metabolic Endotoxemia

Elevated levels of LPS could be the result of increased endotoxin production by a change in gut microbiota; the latter is characterized by decreased proportion of beneficial bacteria (*Lactobacillus* spp., *Bifidobacterium* spp., and *Bacteroides-Prevotella* spp.) to some Firmicutes species (77). Increased intestinal permeability characterized by an increased expression of epithelial tight

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junction proteins such as Zonulin and Occludin are involved in this mechanism. This effect can be completely restored by modulation of gut microbiota. Adam et al., demonstrated that specific changes in gut microbiota composition by feeding arabinoxylans oligosaccharides to obese mice led to an increase in Bifidobacteria and a decrease in Lactobacilli, which consequently improved inflammation and gut barrier integrity. Also, they noticed that the tight junction proteins were upregulated in the colon after the intervention (78).

As mentioned above, probiotics can decrease gut permeability and endotoxemia. The mechanisms for probiotics beneficial effects on barrier function are still unknown.

Probiotics have been shown to produce bacteriocins, which inhibit pathogenic bacteria and regulate intestinal epithelial cells anti-apoptotic and proliferation responses (79, 80).

Moreover, probiotics secrete some proteins that protect intestinal epithelial cell from oxidative stress by inducing cytoprotective heat shock proteins (81). The beneficial activity of probiotics may be exerted through secreting metabolites of lactic acid bacteria. For example, Ménard et al. showed that metabolites of lactic acid bacteria (*B. breve*) may be capable of increasing intestinal barriers function (82). It is noteworthy that LAB products

seem to limit access of LPS to CD14 receptors on monocytes/macrophages. Intestinal macrophages do not express CD14 under basal conditions. This effect was associated with lowered NF- $\kappa$ B signaling in immune cells and decreased inflammation (83). Taken together, two mechanisms may explain probiotics role in the intestinal environment: (1) a direct inhibitory effect on gut permeability, and (2) effect of active bacterial metabolites on epithelial barrier.

Another possible effect of probiotics is restoring the composition of gut microbiota community. Several studies suggest that dysbiosis may contribute to cardiovascular disease risk, and that probiotic supplementation can have favorable effects by normalizing the gut microbiota (84). An irregular profile of gut flora with substantially lower ratio of Bifidobacteria and Lactobacilli to Firmicutes species can affect endotoxin production (85). Also, previous studies have indicated beneficial therapeutic effects of Lactobacillus spp. and other probiotics in patients with CVD (86). In fact, probiotic interventions with Bifidobacteria and Lactobacillus spp. restored numbers of beneficial species and led to a significant decrease in endotoxin levels. Another possible mechanism could arise from the putative role of the Bifidobacterium spp in maintaining the gut barrier. Bifidobacterium spp do not degrade intestinal mucus glycoproteins like other pathogenic bacteria do, and enhance microvillus environment by averting permeability and bacterial translocation (87). It has been shown that products of prebiotics including short chain fatty acids

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(SCFAs) act as an energy substrate for the colonocytes, and have a trophic effect on mucosa which in turn increases villus height and crypt depth, and leads to a thicker mucosal layer in the colon (88, 89). Cani et al. indicated (17) that prebiotic treatment following high fat diet led to higher endogenous GLP-2 production and improvement of the mucosal barrier function, consequently improving tight junctions, decreasing plasma LPS concentrations and reducing inflammatory and oxidative stress. Altogether, these data led the authors to hypothesize that there was a positive correlation between GLP and tight-junction proteins (ZO-1, occludin), and that probiotics may positively impact ME. Further studies are needed to evaluate the effect of different probiotics strains on gut microbiota profile and endotoxemia in subject with CVD.

In vitro models of ME have recently proposed that some probiotics strains such as Lactobacillus rhamnosus and Lactobacillus casei, protect epithelial barrier function against Escherichia coli-induced endotoxemia (90). Moreover, treatment with probiotics induced a variety of changes in the expression of different TLRs. In one study conducted by Schmitz et al., administration of probiotics into the intestine of healthy dogs and those with enteropathies led to increased expression of TLR ligands. In addition, production of TNF $\alpha$  and IL-17A proteins decreased in plasma (91). The gut microbiota can be restored by non-digestible, fermentable carbohydrates, which are known as prebiotics, including inulin, fructooligosaccharide, oligofructose, and xylose; prebiotics consumption leads to selective stimulation of growth and/or activities of beneficial bacteria in colon (92, 93). In this regard, gut microbiota modulation by prebiotic increases bacterial fermentation products, mostly SCFAs, which act as an energy substrate for the colonocytes, subsequently having a trophic effect on mucosa (94). The potential of SCFAs to help form a thicker mucosal layer in jejunum and colon, may explain their effect on decreasing gut permeability and subsequent ME (93). On the other hands, prebiotic intake leads to increased proportion of

beneficial bacteria in the gut microbiome. A recent study demonstrated that administration of prebiotics (oligofructose) could raise *Bifidobacterium* spp. in gut microbiota, which improved gut permeability (68). To additionally support our concept, a summary of studies which found changes in levels of endotoxemia or endotoxin-related markers by prebiotics are presented herein (Table 1). A recent study conducted by Dehghan et al., showed that inulin administration (as prebiotics) for eight weeks, could modulate inflammation and metabolic endotoxemia in women with type 2 diabetes (92). Accepted Article

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Former studies demonstrated that increased *Bifidobacterium* reduced intestinal endotoxin formation, and improved intestinal barrier function through improving intestinal permeability and a GLP-2-dependent mechanism (95). Also, available data have shown that a selective gut microbiota change by increasing endogenous GLP-2 production, contributes to improvement of gut barrier permeability (57). Beside the supposed role of the SCFAs and particular bacterial strains, the precise mechanism underlying the relationship between prebiotic-induced changes in the gut microbiota and enhanced gut barrier function has not been defined yet.

#### CONCLUSION

Human studies have indicated that endotoxemia may lead to inflammation and cardio metabolic consequences. This review reported the potential benefits of prebiotics/probiotics therapy for cardiovascular health, probably by reducing endotoxemia. Although many of these studies have suggested a positive effect of pre/probiotics on ME, we point out that the claim for the favorable effects of these nutraceuticals in cardiovascular diseases is still at its infancy, and requires more comprehensive and well-designed clinical trials. Of particular, evidence from human studies on the association between ME and CVDs is insufficient compared to animal studies. As mentioned above, preliminary evidence suggests that antibiotic therapy suppresses endotoxin and TMAO levels; however, the stability of that effect by long-term use of these agents remains unknown. Therefore, seeking for alternative methods for modulating the gut microbial community, either through food additives or prebiotics/probiotics administration is needed. Further studies are warranted to establish whether prebiotics/probiotics therapies can significantly reduce cardiovascular risk through decreasing ME and metabolite endotoxemia.

#### Material and Methods

To find relevant studies published prior to July 2019, a literature search conducted in the PubMed, Scopus, Embase, Cochrane Library, ProQuest, and Google Scholar electronic databases using the keywords (“probiotic” OR “lactobacillus” OR “bifidobacterium” OR “saccharomyces” OR “Escherichia coli” OR “yeast” OR “prebiotic” OR “inulin” OR “fructooligosaccharide” OR “fructo-oligosaccharide” OR “FOS” OR “galactooligosaccharide” OR “galacto-ligosaccharide” OR “GOS” OR “oligofructose” OR “synbiotic” OR “metabolic endotoxemia” OR “gut microbiota” OR “dysbiosis” OR “gastrointestinal microbiome” OR “lipopolysaccharide” OR “peptidoglycans”) And (“cardiovascular” OR “heart disease” OR “atherosclerosis” OR Accepted Article

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“hypertension” OR “blood pressure” OR “cholesterol” OR “triglycerides” OR “HDL” OR “LDL” OR “hs-CRP” OR “CRP” OR “inflammation” OR “oxidative stress” OR “LPS” OR “TMAO” OR

“TLRs” OR “IL-6” OR “TNF- $\alpha$ ” OR “SCFAs”). The search was limited to English language studies published before July 2019.

#### Eligibility criteria

The eligibility for entering the study were as follows: (1) all clinical trials which evaluated the effect of probiotics and probiotics on the metabolic endotoxemia (i.e. endotoxin) and cardiovascular disease. (2) All animal studies which evaluated the effect of probiotics and probiotics on the metabolic endotoxemia and cardiovascular disease and (1) in vitro models (2) letters, (3) comments, (4) short communications, and (4) studies with insufficient information (e.g., published in non-English-languages or studies that did not provide access to full text) were excluded.

#### Data extraction

The titles and abstracts of the eligible papers were screened independently by two researchers and studies were excluded if they did not meet the eligibility criteria. In the next step, full-text articles were examined based on type of study, study subjects, study design, daily dose, and duration of intervention and main outcome.

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#### Conflict of Interest

The authors declare that they have no conflict of interest.

The probiotic paradox: live and dead cells are biological response modifiers

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Probiotics are usually defined as products which contain viable non-pathogenic microorganisms able to confer health benefits to the host. There are specific gastrointestinal effects of probiotics such as alleviating inflammatory bowel disease, reducing acute diarrhoea in children, inhibiting *Salmonella* and *Helicobacter pylori*, removing cholesterol, secreting enzymes and bacteriocins and immunomodulation. However, many of the effects obtained from viable cells of probiotics are also obtained from populations of dead cells. Heat-killed cells of *Enterococcus faecalis* stimulate the gastrointestinal immune system in chicks. Dead bifidobacteria induce significant increases in TNF- $\alpha$  production.

Administration of heat-killed *E. faecalis* to healthy dogs increases neutrophil phagocytes. The probiotic paradox is that both live and dead cells in probiotic products can generate beneficial biological responses. The action of probiotics could be a dual one. Live probiotic cells influence both the gastrointestinal microflora and the immune response whilst the components of dead cells exert an anti-inflammatory response in the gastrointestinal tract. This is quite analogous to a proposed mode of action of antimicrobial growth promoters in

animal production. This has several implications for the production and application of probiotics, as it will be difficult to assess the relative proportions of live and dead cells in a probiotic culture. Variable amounts of dead cells might contribute to the variation in response often seen with live probiotic cultures. However, the use of dead probiotics as biological response modifiers has several attractive advantages; such products would be very safe and have a long shelf-life.

Probiotics: Live probiotics: Dead probiotics: Gastrointestinal tract: Immunomodulation  
Introduction

There is a widespread interest nowadays in the health benefits of food components or nutrition-based health(1) and probiotics in particular are potentially an important part of a nutrition-based health strategy. Probiotics are usually defined as dietary supplements, containing viable nonpathogenic micro-organisms, which are considered to confer health benefits to the host(2) through their interactions with the gastrointestinal microflora and directly with the immune system. The avoidance and reduction of enteric diseases is a major factor in health maintenance, and the microflora of the gastrointestinal tract are well recognised as playing a fundamentally important role here(3). However, interactions between the gastrointestinal microflora and the host are not always beneficial. A disordered recognition of components of the commensal flora by the host seems most probably a major causal effect of inflammatory bowel disease(4,5).

The pathogenesis of Crohn's disease is now widely accepted as a consequence of an aggressive immune response to the continuous antigenic stimulation by gastrointestinal microflora(6,7). Traditional pathogens are probably not responsible for the onset of Crohn's disease but an increased virulence of commensal bacteria interacting with pathogens stimulates the damaging immune response(7). Therefore, modification of the gastrointestinal microflora by probiotic therapy has therapeutic potential in clinical conditions associated with gastrointestinal barrier dysfunctions and inflamed mucosa. Probiotics may offer a new therapeutic option for the treatment of inflammatory bowel disease as reviewed by Geier et al. (5). Probiotics have been effective in the treatment of acute infectious diarrhoea in children and in the prevention of antibiotic-associated diarrhoea(8–10). There is encouraging evidence for a beneficial effect of probiotics against rotavirus diarrhoea in children(11,12). Probiotics can also be effective in the

Abbreviations: EC-12, commercial product of heat-killed *Enterococcus faecalis*; OVA, ovalbumin; Th1, T helper type 1; Th2, T helper type 2; VRE, vancomycin-resistant enterococci. Corresponding author: Clifford Adams, email Cliff.Adams@skynet.be

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prevention and management of pouchitis, paediatric atopic diseases and the prevention of post-operative infections(8). There are many other beneficial properties of probiotics. They can inhibit *Salmonella* (13–16) and *Helicobacter pylori* (17), remove cholesterol(18,19), and secrete enzymes(20) and bacteriocins(21) into the gastrointestinal tract. Future applications of probiotics in human health have been concisely reviewed by Vanderhoof(22). Probiotics might be useful in controlling inflammatory diseases, treating and preventing allergic diseases, preventing cancer and stimulating the immune system, which may reduce the incidence of respiratory disease.

Modes of action of probiotics

At the cellular level, probiotic micro-organisms have several possible modes of action. They may be able to directly inhibit or kill pathogenic bacteria, and lactobacilli species are often considered to be valuable here(21,23). A second mode of action is that of inhibiting the attachment of pathogens to the wall of the gastrointestinal tract(24). Pathogenic bacteria in the gastrointestinal tract need to attach themselves to the wall of the gut to be able to develop disease syndromes; if probiotic cells can effectively compete with the pathogens for binding sites or bind to the pathogens, the virulence of the pathogens would be reduced. They can have an effect upon host mucosal surfaces, including the mouth, gastrointestinal tract, upper respiratory tract and urogenital tract. Mucosal defence against bacteria is essential for the homeostasis of the host. In the case of the colonised intestine, protection against enteropathogens partly depends upon the indigenous microflora, and probiotics may play a role here. Probiotics have also been implicated in direct interaction with the immune system as reviewed by Isolauri et al. (25). Many probiotic effects are mediated through immune regulation and through the balance of pro-inflammatory and anti-inflammatory cytokines. The population of intraepithelial CD8 $\beta$ T cells was significantly enhanced in piglets treated with a probiotic (*Bacillus cereus* var. *Toyoi*)(26) and human T-cell proliferation was suppressed by *Lactobacillus* strains(3). Consumption of *Lactobacillus plantarum* by healthy humans showed a modulation of NF- $\kappa$ B-dependent pathways, which may be related to the establishment of immune tolerance in healthy subjects(27). There is a wide range of biological responses reported from treatment by various probiotics. These biological responses are also obtained from a diverse range of microbial products. The generally accepted definition of probiotics is that they are live, non-pathogenic microorganisms ingested by the target species which may be human, animal or avian. However, there is considerable published evidence that probiotic preparations comprised of dead cells and their metabolites can also exert a biological response, in many cases similar to that seen with live cells. Consequently, probiotics consisting of either live or dead cells and their metabolites may play an important role in health maintenance and disease avoidance in the host, including modulation of immune responses. This is the crux of the probiotic paradox where both live and dead cells seem to be capable of generating a biological response(28–30).

#### Some problems with conventional probiotics

A major problem in the practical application of probiotics and also in the understanding of their mode of action is that heterogeneous effects are often obtained. This may well be due to the differing ability of the probiotic strains to colonise the gastrointestinal tract. Two strains of lactobacilli, *L. johnsonii* and *L. paracasei*, have been found to have similar in vitro properties(31). However, when they were administered to germ-free mice they colonised the intestinal lumen and translocated into mucosal lymphoid tissues at different densities. The strain *L. johnsonii* colonised the intestine very efficiently at high levels whereas the number of *L. paracasei* cells decreased rapidly and it colonised at low levels. Both strains were able to activate B-cell responses but there were clear differences in patterns of immunoglobulins in the mucosa and in the periphery. Therefore, despite similar in vitro properties, distinct lactobacilli strains may colonise the gastrointestinal tract differently and generate divergent biological responses. Live cells in probiotic products will inevitably lose viability and the actual products will contain varying populations of dead cells(32). The population of dead cells could be even larger than that of live cells but this is frequently not known. This would affect the evaluation of any positive response when the dose was

controlled by the number of live cells. In practice it may not be possible to feed only live bacteria to a subject. There will always be the possibility that an unknown amount of dead cells are being administered with the live cells. Another practical difficulty with probiotics is that ideally they should establish themselves within the gastrointestinal tract and survive in sufficient numbers. However, to do this they need to be of a suitable strain particular to the host animal and this is difficult in practice to achieve, as standardised products must be commercially produced. Furthermore, a probiotic may only achieve a transient colonisation of the gastrointestinal tract. This could arise from a substantial loss of viability of the organisms on passage through the relatively hostile environment of the stomach and small intestine (33,34). The probiotic organisms would have to survive low pH and proteolytic enzymes. However, many probiotic species such as *L. delbrueckii* and *Streptococcus thermophilus* do not readily survive stomach acidity. The recovery of *L. delbrueckii* subsp. *bulgaricus* from the terminal ileum of minipigs was from 0.04 to 0.5%. Recovery rates of total *Strep. thermophilus* were 1.2 and 2.2% (35). Therefore some of the benefits derived from consumption of these probiotics are more likely to accrue from the presence of metabolites or of dead probiotic cells in the gastrointestinal tract (33).

Dead cells as biological response modifiers

Despite the general definition that probiotics are live microorganisms, a variety of biological responses have been reported from administering dead, frequently heat-killed, probiotics to various mammalian and avian species. The preparations of dead cells have also been fractionated and various cellular components shown to produce a range of biological responses.

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**Immunomodulation** A single administration of heat-killed *Enterococcus faecalis* FK-23 preparation was evaluated for immune responses in healthy dogs (36). The probiotic had no effect on the complete blood count or on leucocyte differential count. However, the treatment caused a 1.4-fold increase in neutrophil phagocytic activity compared with non-treated healthy dogs. A single administration of heat-killed probiotic bacteria augmented the host resistance through stimulation of non-specific immune responses *in vivo*. There has been considerable further work on heat-killed *E. faecalis*, which led to the production of a commercial product, EC-12. This is a dried powder of heat-killed cells which has an immunostimulatory function (37). Dietary EC-12 given to newly hatched chicks at 0.05% of the feed stimulated the gastrointestinal immune system and reinforced the immune reaction against a vancomycin-resistant enterococci (VRE) challenge to accelerate its defecation from the chick intestine. As shown in Table 1, the detection frequency (%) of VRE in cloacal swabs of chicks given EC-12 in the feed was lower on days 3 and 7 compared with the control group and with a group given live chicken *Lactobacillus* spp. The EC-12 product is produced from dead cells and there was no treatment with live cells of *E. faecalis*, so no direct comparison between live and dead *E. faecalis* cells can be made. Nevertheless, the preparation of dead cells was clearly able to generate a biological response. Total IgA concentration in the caeca digesta was higher in the EC-12 group than in the control group. Total serum IgG concentration also tended to be higher in the EC-12 treated chicks than in the other treatments. This could be a useful treatment technique, as once VRE are established on a farm it is virtually impossible to eliminate them from animal intestines due to their multiple resistance against antimicrobials (38). Therefore

there is a real need for alternative strategies to prevent infection or to eliminate colonisation of VRE from the gastrointestinal tract of animals. In a subsequent study, administration of dead cells of EC-12 to newly hatched broiler chicks stimulated the production of the antimicrobial peptide,  $\beta$ -defensin(39). This may be one of the major defence mechanisms inhibiting VRE colonisation in young chicks. Both *E. faecalis* and *Lactobacillus* spp. are Gram-positive bacteria and their cell walls are known to stimulate the inflammatory reaction involving macrophages in the mammalian gastrointestinal tract(40–42). They can also induce B-cell activation and stimulate IgA secretion in the intestine(43). This kind of immunostimulation can lead to a rapid removal of the pathogen from the digestive tract in chicks. The relative rapid decline in VRE detection with the EC-12 treatment (Table 1) suggests the involvement of the innate immune system. As EC-12 is derived from dead cells the protective effect of this material is likely to be an immunostimulation. A possible advantage in using a dead cell preparation is that it cannot acquire and transmit the vancomycin (VCM)-resistant plasmid from VRE. There is clearly substantial evidence that heat-killed preparations of *E. faecalis* have an immunomodulating effect in both dogs and chicks(36,37,39). Bifidobacteria are non-pathogenic, Gram-positive organisms that are frequently used in dairy products as a probiotic adjunct. Marin et al. (44) showed that several strains of bifidobacteria had an immunopotentiating activity in clonal murine macrophage and T-cell lines. There was considerable variation in activity among the fourteen different strains of bifidobacteria studied, but four strains used in commercial dairy products were the most stimulatory. This immunomodulation could be elicited by heat-killed bifidobacteria which were able to induce pronounced increases, of up to several hundred-fold, in the production of TNF- $\alpha$  compared with that of controls. IL-6 production also increased significantly. Upon concurrent stimulation of the macrophages with lipopolysaccharide there was substantial increased production of both TNF- $\alpha$  and IL-6 when they were cultured with bifidobacteria. It appears that a direct interaction of bifidobacteria with macrophages enhanced cytokine production. Clearly, heat-killed bifidobacteria are able to act as biological response modifiers although the response seen here was pro-inflammatory. Moderate stimulation of pro-inflammatory cytokines could be beneficial in maintaining a good immunological balance and increasing resistance to infections. However, high concentrations of TNF- $\alpha$  are undesirable(44). There is certainly the potential for dead probiotic cells to have adverse effects and careful screening and selection of strains would still be required. Gastrointestinal epithelial cells secrete a variety of inflammatory cytokines after stimulation by pathogenic bacteria. In particular, IL-6 is produced in response to bacterial infection. This is a multifunctional cytokine involved in diverse biological processes such as the host response to enteric pathogens, acute-phase reactions, haematopoiesis, growth factor for normal or neoplastic cells, and terminal differentiation of B-lymphocytes. This interleukin has traditionally been considered the product of pro-inflammatory cells. However, IL-6 is also known to possess several anti-inflammatory characteristics such as its ability to down-regulate lipopolysaccharide-induced monocyte IL-1 and TNF- $\alpha$  mRNA expression. Both viable and non-viable probiotic cells were able to stimulate IL-6 production in murine small intestine epithelial cells (Table 2)(43). The reduction of IL-6 production when intestinal epithelial cells were treated with live bacteria at a dose rate of  $1 \times 10^8$  cells/ml might be due to a diminution in pH of the

culture medium by the metabolically active bacteria. This is consistent with the results for heat-killed bacteria

Table 1. Vancomycin-resistant enterococci detection (%) in cloacal swabs of broiler chicks treated with a conventional live probiotic or with dead probiotic cells of *Enterococcus faecalis* (EC-12) (Sakai et al. (37))

Age of chicks (d)

Treatment Chicks (n) 1 3 7

Control 13 100 100b 77a,b Lactobacillus (live cells) 6 67 100b 100b EC-12 (dead cells) 13 46 31a 38a

a,b Percentages within a column with unlike superscript letters were significantly different ( $P < 0.05$ ).

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where IL-6 production increased as the bacterial load increased (Table 2). Chuang et al. (45) showed that three heat-killed strains of *Lactobacillus* were able to modulate immune responses by stimulating proliferation of murine splenocytes. In addition, these heat-killed probiotic cells also stimulated high-level secretion of IL-12 p70 in dendritic cells of mice and they switched T helper cells to T helper 1 (Th1) immune responses. Components of lactobacilli cells have the potential to play an important role in modulating immune responses and allergic reactions but this does not depend on the cells being alive. Both live and heat-killed *Lactobacillus* GG had an anti-inflammatory effect in rats suffering from experimental arthritis (46). This is similar to the oral treatment with live *L. reuteri* that significantly attenuated an allergic airway response in mice (47). Clearly the anti-inflammatory effect of these probiotics did not depend upon the viability of the microorganism. In stark contrast to these results, Ma et al. (48) reported that live cultures of *L. reuteri* were essential for an inhibitory effect on expression of IL-8 in human epithelial cell lines. IL-8 is an important mediator of the innate immune system. The pro-inflammatory cytokine TNF- $\alpha$  induces IL-8 production and this could only be suppressed by live probiotic cells. Neither heat-killed nor  $\gamma$ -irradiated cells were able to generate a response. This exemplifies the difficulty of drawing a general consensus on the action of probiotics. In these two studies with *L. reuteri* different experimental models were used, mice (47) v. an in vitro model with human epithelial cells (48). There has been a considerable amount of work published on the interactions of probiotics and TNF- $\alpha$  and other cytokines. The probiotic *L. rhamnosus* GG modulated the TNF- $\alpha$ -induced release of IL-8 from Caco-2 cells (30). High doses of live *L. rhamnosus* GG in the absence of TNF- $\alpha$  actually induced the production of IL-8. Heat-killed probiotics also reduced IL-8 production from TNF- $\alpha$ -induced cells. However, by themselves, dead cells caused only a small increase in IL-8 production. This suggests that heat-killed probiotics may be able to prevent intestinal inflammation without the potential pro-inflammatory effect exhibited when the intestinal epithelium is exposed to high quantities of *L. rhamnosus* GG. Further work with TNF- $\alpha$  showed that six heat-killed *Lactobacillus* strains had a pro-inflammatory effect in inducing the secretion of TNF- $\alpha$  from mouse splenic mononuclear cells (49). However, there was a clear difference among strains. The most active *L. rhamnosus* induced approximately four times more TNF- $\alpha$  than the least active *L. casei*. It appears that the *Lactobacillus* strain *L. rhamnosus* is particularly effective in inducing the production of TNF- $\alpha$ . Furthermore, these examples suggest that both live and dead probiotic cells may have an effect outside

the gastrointestinal tract, as reviewed by Lenoir-Wijnkoop et al. (50). A potential problem with live probiotic cells is that they may cause some pathology of their own, particularly in severely immunodeficient patients. However, the use of both heat-killed *L. acidophilus* and *L. casei* was able to induce some limited protection against infection with *Candida albicans* in immunodeficient mice(51). In mice that had been challenged by *C. albicans*, treatment with heat-killed probiotics suppressed orogastric candidiasis 2 weeks after colonisation and also suppressed the number of viable *C. albicans* cells in the gastrointestinal tract. Dead cells of these probiotics did not exacerbate any problems and were able to offer some limited protection against candidiasis in immunodeficient mice.

Colitis Experimental colitis as a disease model can be induced in Wistar rats by treatment with indomethacin(52). Treatment with a proprietary probiotic administered either orally or subcutaneously had an anti-inflammatory effect and prevented gastrointestinal lesions. The response was obtained with both live and dead probiotic cells. Supplying dextran sodium sulfate in the drinking water can also induce experimental colitis in mice and this is another useful model to study the effect of probiotics(53,54). The severity of this experimental colitis was attenuated by either non-viable g-irradiated, or by viable probiotics, but not by heat-killed probiotics in this instance (Table 3)(53). The probiotics used here were a commercial preparation containing four strains of lactobacilli, three strains of bifidobacteria and one strain of *Strep. salivarius* subsp. *thermophilus*. Further investigations showed that the experimental colitis was ameliorated by the administration of probiotic DNA(53). However, methylated probiotic DNA, calf thymus DNA, and DNAase-treated probiotics had no effect. These results suggest that the protective effects of probiotics in this instance were mainly mediated by their own DNA rather than by their metabolites or their ability to colonise the colon. Heat treatment may have denatured the DNA so that heat-killed cells were no longer active.

Table 3. Effect of viable and dead probiotics on experimental colitis in mice induced by treatment with dextran sodium sulfate (Rachmilewitz et al. (53))

Treatment Disease activity score

None (control) 8.0 Viable probiotics 2.7\* Irradiated probiotics 0.1\* Heat-killed probiotics 7.0

\* Significantly different from control (P, 0.05).

Table 2. The effect of viable or heat-killed *Lactobacillus casei* and *L. helveticus* on the production of IL-6 (pg/ml) by murine small intestine epithelial cells (Vinderola et al. (43))

Dose of probiotic Treatment None 106 107 108

Negative control 395 – – – Positive control (LPS at 0.1mg/ml) 890\* – – – *L. casei* (viable) – 530\* 520\* 300\* *L. helveticus* (viable) – 480\* 620\* 220\* *L. casei* (heat-killed) – 400 500\* 620\* *L. helveticus* (heat-killed) – 580\* 650\* 720\*

LPS, lipopolysaccharide. \* Significantly different from negative control (P, 0.05).

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Oral administration of *Bacillus polyfermenticus* by oral administration protected mouse colonic mucosa from inflammatory responses due to colitis induced either by dextran sodium sulfate or by 2,4,6 trinitrobenzenesulfonic acid(54). The biological responses seen here both with live and dead cells are a result of the probiotic interacting with the gastrointestinal mucosa rather than with the microflora. It is molecular components of the probiotic that generate the biological response and this could probably be achieved with

either live or dead cells. The most likely mode of action is through stimulation of Toll-like receptors which exert an immunomodulatory effect in the gastrointestinal tract(53,54). Modulation of pain response Live, heat-killed or g-irradiated *L. reuteri* or medium containing metabolites from the bacteria were orally administered to rats suffering visceral pain induced by colorectal distension(55). The probiotic treatment inhibited the pain response to colorectal distension through effects on enteric nerves. This is another example where dead probiotic cells seem to have an effect outside the gastrointestinal tract.

Allergic diseases Allergic diseases such as atopic dermatitis, atopic eczema and allergic rhinitis are serious social problems in many countries(29). These allergic diseases are characterised by an elevation in serum IgE levels(56), which in turn is generally thought to be caused by a skewed balance between Th1 and T helper type 2 (Th2) cells(57). The balance of the two types of cells is considered to be important to maintain homeostasis of the host. *L. casei* strain Shirota was killed by heating and lyophilised before being used to treat mice that had been injected intraperitoneally with ovalbumin (OVA) to induce IgE production(57). Treatment with the heat-killed *Lactobacillus* reduced total IgE production in mouse serum. This suggests that administration of heatkilled cells of *L. casei* strain Shirota could be a treatment against allergic diseases. A total of fifty-nine strains of heat-killed *L. brevis* were examined for their ability to induce IL-12 and interferon-g from mouse Peyer's patches cells(58). Strains which were selected for their ability to induce a strong Th1 immune response inhibited both total IgE and antigen-specific IgE production and improved the Th1/Th2 balance by enhancing IL-12 and interferon-g and inhibiting IL-4 production from OVA-sensitised mouse splenocytes. These in vitro results were followed by animal trials using mice(58). A particular strain of *L. brevis*, SBC8803, was fed to OVA-sensitised mice at 0.5% of the diet for 4 weeks. Total and OVA-specific IgE in the serum of mice which were fed the heat-killed bacterium was significantly lower than that of the control mice. The interferon-g/IL-4 value, which represents the Th1/Th2 balance, from splenocytes from the mice fed the *L. brevis* was significantly higher than that seen in the splenocytes from the control mice not fed the *L. brevis*. The results shown here for *L. casei* Shirota(57) and *L. brevis* (58) were probably due to the heat-killed probiotics improving the Th1/Th2 balance in favour of a predominance of Th1 cells. In a subsequent study, twenty strains of heat-killed lactobacilli isolated from human subjects were screened for their stimulatory activity to produce cytokines by murine splenocytes in vitro and by their ability to suppress IgE production when they were orally administered to allergic mice(29). One strain of *L. gasseri* had a higher stimulatory activity of the cytokine IL-12 production than the other lactobacilli tested. Oral administration of *L. gasseri* was more effective in reducing the serum antigen-specific IgE levels in OVA-sensitised mice compared with other lactobacilli and the control (Table 4). Furthermore, the stimulatory activity for IL-12 production was reduced after treating the lactobacilli with N-acetyl-muramidase and tended to be positively correlated with the amount of peptidoglycan in the cells. Several different reports indicate that heat-killed lactobacilli strains – *L. casei* strain Shirota(57), *L. brevis* (58) and *L. gasseri* (29) – can improve the Th1/Th2 balance and inhibit IgE production. Clearly, dead probiotic cells can act as biological response modifiers for allergic diseases.

Reduction of cholesterol Another effect of probiotics is that of reducing cholesterol. Eleven strains of lactobacilli were able to remove between 31 and 97% of cholesterol from the medium, so this phenomenon may be a general effect of probiotics(18). Again, both live and

heat-killed cells were able to remove cholesterol. Cholesterol removed by dead or resting cells ranged from 0.79 to 3.82mg/g of dry weight compared with growing cells, which ranged from 4.53 to 16.03mg/g of dry weight. Heat-killed cells of lactococci and of lactobacilli were also able to take up cholesterol although at a lower rate than living cells. This is probably due to binding to the cell surface(18,19). Strains of lactococci and of lactobacilli probably remove cholesterol by two mechanisms. One is through assimilation of cholesterol by living cells and two is by binding to the cell walls. Adhesion to the cell surface could occur in both living and dead cells and therefore dead cells may be promising candidates as a dietary supplement to lower serum cholesterol.

Table 4. Effect of oral administration of heat-killed lactic acid bacteria on serum IgE level in ovalbumin-sensitised mice as a model for allergic disease (Sashihara et al. (29))

Lactobacilli strain

Serum IgE (arbitrary units/ml)

None (control) 1850 Lactobacillus crispatus JCM 1185T 1500 L. plantarum JCM 1149T 1600 L. plantarum JCM MEP 170402 2200 L. gasseri JCM 11313T 950 L. gasseri MEP 170407 960 L. gasseri MEP 170413 1010 L. gasseri OLL 2809 800\*

\* Significantly different from control (P , 0.05).

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Activities of probiotic cell fractions

There is considerable evidence that not only dead cells, but also metabolites and cell fractions of probiotics can exert a biological response. Various microbiological components such as cell homogenates(59,60), b-glucans(61), teichoic and lipoteichoic acids(49), peptidoglycans, lipopolysaccharides and DNA(53,62) have an immunomodulating effect, probably through stimulating the innate immune system. Heat-treated homogenates were prepared from various probiotic bacteria; *L. rhamnosus* GG, *Bifidobacterium lactis*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* (59). All the homogenates suppressed phytohaemagglutinin-induced proliferation of human blood peripheral mononuclear cells. When the proliferation assays were repeated with cytoplasmic and cell-wall extracts derived from the homogenate of *L. rhamnosus* GG, the cytoplasmic extract, but not the cell-wall extract, was suppressive(59). These bacteria possess a heat-stable antiproliferative component that is not associated with the cell wall. Probiotic-mediated biological responses have also been demonstrated in fish cells. Two teleost fish cell lines SAF-1, a fibroblast cell line, and epithelioma papulosum cyprini (EPC), an epithelioma from carp, were used(60). These cells rapidly proliferate and biological responses were assayed as an anti-proliferative effect and the induction of apoptosis. The cells were grown in the presence of cytoplasmic extracts from two probiotic strains of *L. delbrueckii* subsp. *lactis* (60). The SAF-1 cells were more susceptible to growth inhibition by the probiotic extracts than were the EPC cells.

Apoptosis took place following growth inhibition, which indicated that cytoplasmic extracts of probiotic bacteria were able to exert an immunological response in cultured fish cells. These studies(59,60) are a good model for the responses of commensal and pathogenic bacteria in the gastrointestinal tract. Generally, bacteria and bacterial homogenates of the commensal gastrointestinal microflora do not stimulate proliferation of mononuclear cells(63) and this plays an important role in the maintenance of hyporesponsiveness to foreign antigens(64). Pathogens, in contrast, stimulate the proliferation of mucosal immune

cells and this produces an inflammatory reaction(63,65). Cell contents from probiotic bacteria suppress immune responses invitro in human blood mononuclear cells and in cells from fish, suggesting that this may be a widespread response. This has interesting possibilities for the further development of probiotic-based products. Non-viable cell extracts would probably have a long shelf-life and be easier to store in commercial practice. Both crude extracts and purified lipoteichoic acids from *L. casei* and *L. fermentum* could significantly induce TNF- $\alpha$  secretion from mouse splenic mononuclear cells(49). This suggests that purified lipoteichoic acids may be a better candidate for clinical use than whole bacteria since they do not contain other bacterial components which might cause side effects. In mice with experimentally induced colitis, DNA from a commercial probiotic mixture ameliorated the severity of the disease. Methylated probiotic DNA, calf thymus DNA and DNase-treated probiotics had no effect(53).

Table 5. The effect of dietary  $\beta$ -glucans on the concentrations of various cytokines induced by lipopolysaccharides (LPS) in the plasma of pigs (Li et al. (67)) (Mean values with their pooled standard errors)

b-Glucans at 0ppm b-Glucans at 50ppm

Cytokine Time (h) No LPS LPS at 25mg/kg body weight No LPS LPS at 25mg/kg body weight SEM

IL-6 (pg/ml) 0 149 148 135 149 8.9 3 164 415 142 349 25.6 TNF- $\alpha$  (ng/ml) 0 0.61 0.55  
0.50 0.45 0.04 3 0.53 5.94 0.48 4.67 0.53 IL-10 (pg/ml) 0 85 84 92 97 4.43 3 89 105 105  
127 5.06

ppm, Parts per million.

Table 6. Biological responses to dead cells of various probiotics

Biological response References

Ameliorate arthritis Baharav et al. (2004)(46) Anti-inflammatory effect Zhang et al. (2005)(30), Chuang et al. (2007)(45) Attenuate colitis Laudanno et al. (2006)(52), Rachmilewitz et al. (2004)(53), Imet al. (2009)(54) Attenuate visceral pain Kamiya et al. (2006)(55) Cholesterol binding Kimoto et al. (2002)(18), Liong & Shah (2005)(19) Pro-inflammatory effect, inducing TNF- $\alpha$  secretion Marin et al. (1997)(44), Matsuguchi et al. (2003)(49), Lenoir-Wijnkoop et al. (2007)(50) Reduce allergic diseases through reduction of IgE Sashihara et al. (2006)(29), Matsuzaki et al. (1998)(57), Segawa et al. (2008)(58) Reduce IL-8 production from TNF- $\alpha$ -induced cells Zhang et al. (2005)(30) Stimulate gastrointestinal immune system against VRE Sakai et al. (2006)(37), Sakai et al. (2007)(39) Stimulate IL-6 production Marin et al. (1997)(44) Stimulate proliferation of splenocytes Chuang et al. (2007)(45) Suppress mononuclear cell proliferation Pessi et al. (1999)(59), Salinas et al. (2008)(60) Systemic effects Wagner et al. (2000)(51), Kamiya et al. (2006)(55)

VRE, vancomycin-resistant enterococci.

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Oral treatment of mice with probiotic DNA resulted in a reduction in mucosal secretion of TNF- $\alpha$  and an improvement in histological disease(62). It is interesting here to note that the probiotic DNA was effective when given orally. The possibility of oral dosing is a big advantage for the practical application of probiotics. In these examples also the protective effects of the probiotics were mediated by their DNA rather than by an ability to colonise the gastrointestinal tract. Extracellular polysaccharides from *L. delbrueckii* ssp. *bulgaricus*

OLL1073R-1 were able to generate an immunomodulatory response(66). Fractionation of these polysaccharides yielded an active high-molecular-weight acidic phosphopolysaccharide containing D-glucose and D-galactose. Stimulation of mouse splenocytes by this acidic polysaccharide significantly increased interferon-g production. Oral administration of the phosphopolysaccharide to mice augmented natural killer cell activity. Production of the active phosphopolysaccharide was strain specific in that *L. bulgaricus* OLL1256 and *Strep. thermophilus* OLS3059 did not have an immunomodulating effect. The b-glucans in particular have been referred to as biological response modifiers(61), but this term can be applied to many different components of microbial origin. Such responses clearly have nothing to do with live or viable probiotic preparations. b-Glucans extracted from the cell walls of *Saccharomyces cerevisiae* are able to potentiate the immune system. A dose rate of 50 parts per million (ppm) was able to improve the growth rate of pigs(67). The mode of action may be related to immune responses, as the b-glucans at 50ppm partially suppressed increases in plasma concentration of IL-6 and TNF-a brought about by a lipopolysaccharide challenge (Table 5). There was an increase in IL-6 up to 415ng/ml without b-glucans, but only 349ng/ml with b-glucans. Similarly, the increase in TNF-a was reduced from 5.94 to 4.67ng/ml. Conversely, b-glucans enhanced the increase in plasma concentrations of IL-10. The cytokines IL-6 and TNF-a are pro-inflammatory and they not only modulate immunity but can also directly regulate nutrient metabolism and cause detrimental effects upon animal performance. IL-10 is an anti-inflammatory cytokine that suppresses the activity of the transduction of NF-kB, which is a major transcription factor of proinflammatory cytokines. Therefore, if feeding b-glucans promotes secretion of anti-inflammatory cytokines such as IL-10 and decreases secretion of pro-inflammatory cytokines such as TNF-a and IL-6, then less activation of the immune system would be achieved, which could result in improved growth performance.

### Conclusions

It is clear that both live and dead cells in probiotic products can generate a wide range of biological responses(8). The specific biological responses generated by dead probiotic cells are summarised in Table 6. These responses have been obtained from in vitro cell-culture studies and from in vivo studies with mice, rats, dogs and chickens. Many of the biological responses found with both live and dead probiotics are not antimicrobial effects but are, rather, immunomodulating effects. Frequently they seem to exert an anti-inflammatory effect upon the cells of the gastrointestinal tract. This is quite analogous to the proposed mode of action of antimicrobial growth promoters in animal production(68). Antimicrobial growth promoters have for many years proved to be effective in improving growth and feed efficiency in species such as pigs and poultry. These antimicrobial growth promoters are in fact antibiotics added to the feed of animals in low sub-therapeutic amounts. Despite their widespread use over many years the precise mode of action of antimicrobial growth promoters has never been conclusively elucidated. It seems, however, that an antibiotic mechanism for the mode of action of antimicrobial growth promoters is unlikely and that their target is not the microflora in the gastrointestinal tract. Most antibiotics also have a nonantibiotic, anti-inflammatory effect upon the cells of the gastrointestinal tract. They attenuate the inflammatory response. Consequently, the levels of pro-inflammatory cytokines would be lower than those in untreated animals. The effect of probiotics could thus be a dual one where live probiotic cells might well influence the gastrointestinal

microflora and have an immunomodulating effect, whereas the components of dead cells could exert an anti-inflammatory response (Fig. 1). However, the relative importance of these two effects is difficult to assess since an immunomodulating response of both live and dead probiotic cells has been extensively investigated. Dead probiotic cells are not a necessary requirement to generate a biological response but they may be sufficient. Therefore the positive effects shown by both live and dead probiotic preparations illustrate the probiotic paradox. The findings that live probiotics may not be mandatory to be beneficial could have a major impact on the practical use and manufacturing of probiotics(28). It will be difficult to assess the proportion of dead cells in a viable probiotic culture. Consequently, variable amounts of dead cells might well contribute to the variation in response often seen with live probiotic cultures. Conversely, it is relatively easy to demonstrate that cultures of killed probiotic products would not contain any live cells. Products based on dead cells would be relatively easy to standardise and would have a long shelf-life. Also the use of dead cells would permit a wider range of micro-organisms to be considered as probiotics. Clearly with live cultures, only well-recognised

Live and dead cells

Live cells

Probiotics as food components

Gastrointestinal mucosa (inflammatory response)

Gastrointestinal microflora (modify)

Fig. 1. Proposed dual function of probiotics where live and dead cells interact with the gastrointestinal mucosa and live cells also interact with the gastrointestinal microflora.

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non-pathogenic species can be used. Products based on dead cells could be produced from many species of microorganisms. The use of dead probiotics as biological response modifiers has several other attractive advantages. There is always the risk that live probiotic bacteria could cause some pathology of their own. Severely immunodeficient subjects may be at risk from treatment with live probiotics and therefore the use of dead cells would be a safer alternative. It is likely that dead cells would not suffer from the low pH conditions in the stomach. It may also be possible to produce microbiologically non-viable yet immunologically active probiotic food products that are easier to store and have a long shelf-life. The biological response-modifying activity of dead probiotic cells is clearly somewhat similar to an oral immunisation response from a vaccine. In calves use of a live vaccine based on *Salmonella typhimurium* gave excellent protection against a challenge infection by a virulent strain of *S. typhimurium* (69). Oral immunisation of mice with a vaccine based on killed *S. typhimurium* was also quite effective(70). A vaccine prepared from formalin-killed cells of enterotoxigenic *Escherichia coli* cells induced a strong immune response in human subjects(71). Similarly, an oral dose of inactivated whole-cell *Pseudomonas aeruginosa* vaccine given to human volunteers had no adverse safety implications(72). There was also a significant increase in intracellular macrophage killing of opsonised *P. aeruginosa* in the presence of the post-vaccination sera. There is substantial evidence from in vitro studies and animal studies that both live and dead probiotic cells can act as biological response modifiers. These observations suggest that a food supplement based on dead probiotic cells could represent an intermediate stage between an oral

vaccine and a classical live probiotic. Such a product could have applications both in animal husbandry and in human health.

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#### Executive Summary

##### Rationale

A variety of probiotic supplements are now available for human use in the UK. These range from fermented milks to lyophilised forms, containing both single and multiple strains.

However, there is an almost total absence of comparative data on probiotic effects within the

human gut and their inherent safety implications. This project aimed at providing independent

information on the effects of existing probiotics in the alimentary tract. The project was designed to address the following issues:

- To compare commercially available probiotic strains characterised and identified in an independent non-FSA funded study.
- To test the survival of probiotics in the gastrointestinal milieu
- To determine whether probiotics affect the composition of the gut microflora

##### Summary of the approach and objectives

The approach used several validated in vitro models simulating the physico-chemical events

arising in the stomach, upper intestine and lower intestine. A wide number of strains were isolated from commercial probiotic products and phylogenetically identified. Only probiotic

strains originating from products which label matched their microbiological content were chosen for the study. All selected probiotics were treated with digesta resembling the gastric

environment. Probiotic strains that were able to survive the stomach conditions were subsequently considered for testing their survival in the upper intestine. This exercise determined which probiotics had the capacity to survive transit to the lower intestine– the target organ for probiotic residence and effects. Survival in the colon was tested in an in vitro

model of the human large intestine that reflects microbial events in the ascending, transverse

and descending regions of the colon. The effect of probiotic on gut microbial balance was also

investigated using a molecular methodology.

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How closely were the objectives met?

All tasks agreed in the proposal were accomplished. Valuable information on the microbiological effect that probiotics exert were obtained from the study. The project has

clarified the most robust/reliable products for gut survival particularly for *Lactobacillus* spp.

Further studies would need to clarify the effect of *Bifidobacterium* spp. and *Enterococcus faecium* in the lower intestine.

Outline of the main findings

Microbial strains originating from eight different commercial products were isolated and sequenced. Of the products tested, not all the products displayed a profile of probiotics similar

to what was stipulated on their labelling. A collection of 35 bacterial strains was gathered from

the initial screening. Principal strains isolated from commercial products were from *Lactobacillus* spp. and *Bifidobacterium* spp.

The survival of thirty two probiotic strains was assessed in simulated gastric contents.

After

twenty minutes incubation in the presence of simulated gastric contents at pH varying between 1 and 3, *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* sp. and *Lactococcus lactis* sp. showed a good survival in the gastric environment. A ratio was calculated to estimate the proportion of bacterial cells surviving the gastric milieu.

Eighteen

strains had a ratio of live cells greater than 0.5 at pH 2 or 3 or at both pHs indicating that 50%

or more probiotic cells were still viable after 20 minutes in the stomach environment.

On the basis of their resistance to simulated gastric content, the strains selected in the stomach challenge were tested for their tolerance to bile acids. The growth of probiotic strains

in MRS broth in the absence or in the presence of bile acid salts was monitored at two concentrations of bile acids 18g.L<sup>-1</sup> and 36g.L<sup>-1</sup>. A coefficient of growth inhibition (C<sub>inh</sub>) was

calculated for each bile concentration to characterise the effect of bile acids on probiotic growth. Probiotic strains were classified in three groups according to their coefficient of inhibition. Six strains showed a C<sub>inh</sub> close to zero indicating little or no effect of the presence

of bile salts on the growth of probiotic. Seven strains had their growth slowed down by bile

acids ( $0.2 < C_{inh} < 0.4$ ). Five strains showed a poor tolerance to bile acid content as shown by a

C<sub>inh</sub> greater than 0.4 and the absence of growth. The strains of the latter group were unlikely

to reach the large intestine intact. A sub-set of strains was challenged with simulated upper intestine contents containing pancreatic enzymes. In this test, *Lactobacillus* spp. showed a higher sensitivity to upper intestinal content than predicted from the bile acid tolerance test.

On the other hand, *Bifidobacterium* sp. and *Enterococcus faecium* showed a good survival in

this environment. *Lactococcus lactis* did not, however, survive well in both assays.

Six *Lactobacillus* strains were subsequently selected for test of survival in the lower intestine.

The strains studied were *L. casei immunitass*, *L. casei shirota*, *L. plantarum*, *L. pentosus*, *L. reuterii*, *L. acidophilus subsp johnsonii* and *L. delbrueckii subsp bulgaricus*. Each strain was studied independently. *L. casei shirota*, *L. plantarum*, *L. pentosus* and *L. reuterii* were able to survive in measurable level for five days after inoculation of the strain. *L. casei immunitass*, *L. delbrueckii subsp bulgaricus* and *L. acidophilus susp. johnsonii* displayed poor survival. Results of the interaction with the residential microflora showed that addition of probiotic did not affect significantly the total number of bacteria growing in the continuous culture. Little difference were also seen on the levels of main bacterial species numerated by Fluorescent In Situ Hybridisation. Variability in the composition of the microflora was seen from one gut model run to another but *Bacteroides* population remained at high levels throughout the 10 days of sampling in all gut models. Bifidobacteria were generally less prevalent at the end of the wash-out period. Although probiotic from *Lactobacillus* spp. were added to the three stage fermentation system, *Lactobacillus* group remained at sub-dominant level in the three vessels of the fermentor.

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## Introduction

### Background to the project

Dietary modulations of the human gut microflora can be of great benefit to health. The most frequently used dietary method of influencing the gut flora composition is the consumption of probiotics. Over the years many species of micro-organisms have been used. They consist mainly of lactic acid bacteria (*lactobacilli*, *streptococci*, *enterococci*, *lactococci* and *bifidobacteria*). *Bacillus* spp. may also be used.

The project aims at comparing the persistence of probiotics within the human gastrointestinal

tract. Probiotics are marketed as health or functional foods whereby they are ingested for their

purported positive advantages in the digestive tract and/or systemic area. To exert any beneficial properties, probiotics must have robust survival properties in the gut. Given their

wide use in the UK, purported health aspect and the fact that they contain living organisms, all

new probiotics would be subject to a rigorous pre-market safety evaluation under the terms

and conditions of the Novel Foods Regulation (EC) 258/97 before they can be sold in the EU,

and all existing probiotic products must comply with the Food Safety Act (1990).

Scientific principle and rationale behind the choice of approach

It is important that reliable and extensive independent data are generated on product survival.

Colonisation of the human gut may be prevented by the natural resistance exerted by the commensal gut microflora therefore compromising any effect of the probiotic strain. The work

suggested here built on pre-existing information on genetic integrity of commercial products

available in the UK markets. Various products have previously been tested for their probiotic

content and the isolated strains phylogenetically identified. Products where the probiotic strains matched their label were subjected to a rigorous comparative assessment of effects in

the gastrointestinal tract. In vitro approaches were chosen for this study to allow standardisation of testing conditions and direct comparisons between probiotic strains.

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Aims and objectives

The various facets that were addressed were: survival in the gastric, small and large intestinal

regions and effects on the colonic microflora balance. As extensive studies covering the effect

of all existing probiotics in each intestinal regions would be time-consuming, the strategy adopted here was a selective screening. Only probiotic strains showing a positive response in

the considered digestive compartment were selected for assessment in the next digestive compartment. Thus, a decrementing number of probiotic organisms was studied at each stage of the project.

Material and Methods

Bacterial isolation of strains from commercial products

Twelve commercial probiotic products were selected from the supermarket shelves and health food shops. One gram of product was homogenised in 9 ml of Phosphate Buffer Saline

(PBS, Oxoid, UK). Serial 10-fold dilutions were subsequently prepared in anoxic diluents. Diluents contained half strength peptone water (7.5 g.L<sup>-1</sup>, Oxoid, UK) and L-cysteine HCL (0.5

g.L<sup>-1</sup>, BDH, UK) adjusted at pH 7.0. Selected dilutions (10<sup>-2</sup> to 10<sup>-6</sup>) were plated in duplicate

on four growth media: Wilkins-Chalgren (WC) agar (Oxoid, UK), Raffinose Bifidobacterium agar (Hartemink et al, 1996), Beerens agar (Beerens et al, 1996), Rogosa agar (Oxoid, UK).

Plates were incubated anaerobically for 48h at 37°C. Colonies with different phenotypes were

selected and sub-cultured on WC agar plates until pure strains were obtained. Isolated strains

were maintained on cryogenic beads in Microbank tubes at  $-70^{\circ}\text{C}$ . For later experiments, probiotic strains were revived on Mann, Rogosa and Sharp agar (MRS agar, Oxoid, UK) and sub-cultured on MRS broth (Oxoid, UK) in anaerobic conditions.

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Bacterial characterisation by genotyping prior to this project starting

Isolated probiotic strains were identified by amplifying and sequencing the hyper-variable regions of the bacterial 16S rRNA. The nucleotidic sequence obtained was compared to sequences stored in databases to ascertain identity.

DNA extraction and phylogeny

Isolates were revived on Wilkins-Chalgren anaerobes agar. DNA was extracted using a phenol/chloroform technique (Lawson et al., 1989). 16s rRNA gene was amplified by 34 cycles Polymerase Chain Reaction (PCR) using universal 16s rRNA gene forward primer pA (Eden et al., 1991) and reverse primer pE (Edwards et al., 1989).

PCR amplification was carried out using PCR AmpiTaq<sup>®</sup> kit. PCR cycling was performed on a

Gene Amp PCR system 9600 thermocycler (Cetus, Perkin-Elmer). A QIA-quick PCR purification kit (QIAGEN Ltd, West Sussex, UK) was used for purification of the DNA strands. A portion of the PCR product proximal to the 5' end of the rRNA was sequenced (~ 500 nucleotides). ABI PRISM<sup>®</sup> dRhodamine terminator cycle sequencing kit with the AmpliTaq DNA polymerase FS (PE Applied Biosystems Inc., California) and an automatic DNA sequencer (model 373A; PE Applied Biosystems Inc.) were used. Initial screening of the isolates was carried out using reverse primers pD\* and  $\delta$  (Lawson et al., 1989)

Sequences generated were compared to bank collection using FASTA. Identification was determined on the basis of >97% homology to the most closely related sequence.

The output from this earlier study was required to select appropriate commercial strains.

The

initial objective was therefore "Identify products to be tested" and Task 01 was "Selection of probiotic products".

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Survival in simulated gastric content

Overnight culture of probiotics were submitted to pH values ranging from 1- 3. A control treatment was run at pH 6.5. Pepsin (Sigma, UK) was added to the experimental medium at all pHs to simulate the activity of gastric enzyme. The number of live probiotic cells was assessed by plating out tested suspensions on MRS agar at T0 and 20 min. Simulated gastric

juice (SCJ) medium was made of peptone water (7.5 g.L<sup>-1</sup>) dissolved in distilled water and adjusted at pH 1, 2, 3 or 6.5 using 1 M Hydrochloric acid (BDH, UK). SCJ medium was aliquoted in Hungate tubes (9.5 ml per tubes), reduced anaerobically and autoclaved. Prior to

the experiment, a fresh solution of pepsin (300 mg.L<sup>-1</sup>) was made up in 0.5 ml simulated gastric juice, incubated at  $37^{\circ}\text{C}$  for 5 min and added to Hungate tubes (final concentration 15

mg.L<sup>-1</sup>). Pure cultures of probiotics were grown anaerobically overnight in 10 ml of MRS broth.

The inoculum was concentrated by centrifugation at 2000 rpm. The pellet was re-suspended

in 1 ml of SCJ taken from Hungate tubes, the mixture was immediately transferred in the corresponding Hungate tubes. At T 0 and 20 min, one millilitre sample was taken and diluted

serially eight times in peptone water (14 g.L<sup>-1</sup>, pH 6.5). Dilutions 1 to 8 were plated out on WC

agar in duplicates. Plates were incubated for 48h in anaerobic cabinet at 37°C before numeration. To compare bacterial survival, a ratio was calculated as follows:

$r = \text{Average of cells at T20} / \text{Average of cells at T0}$ .

If  $r=1$ , bacterial survival was not affected by the simulated gastric environment. A ratio equal

to 0.5 indicated a loss of half the viable cells present in the culture. A ratio greater than one indicated bacterial growth. The threshold for selection of bacterial strains for subsequent tests

was fixed at  $r=0.5$ .

**Bile acid tolerance**

The rapidity of growth in a broth medium with and without bile acids was assessed using a validated method (Charteris et al., 1998). One millilitre of overnight culture was inoculated in

Hungate tubes containing 9 ml MRS broth supplemented with 18 g.L<sup>-1</sup> or 36 g.L<sup>-1</sup> oxgall (Sigma, UK). Non-supplemented MRS broth was used as control treatment. Hungate tubes were incubated anaerobically at 37°C for 8 hours. Cultures were monitored hourly for growth

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by measuring absorbance of the culture medium at 650 nm. Coefficient of inhibition was calculated using a method adapted from Gopal et al. (2001) as follows:

$C_{inh} = (\Delta T8-T0 \text{ Control} - \Delta T8-T0 \text{ Treatment}) / (\Delta T8-T0 \text{ Control})$

Where  $\Delta$  represented the difference in absorbance between T0 and T8. The experiment was done in triplicate. Only strains achieving a  $C_{inh}$  smaller than 0.40 at both concentrations of bile

acids were considered for further experiments.

**Survival in simulated upper intestine content**

The survival of probiotic strains to simulated upper intestine content was achieved by subjecting the probiotic cells to a micro-aerophilic alkaline medium containing bile salts and a

mixture of pancreatic enzymes. The survivability of the cells was measured before and after 2

hours of incubation at 37°C. Simulated upper intestine juice (UIJ) consisted in Bile salts no 3

(1 g.L<sup>-1</sup>, Oxoid, UK) suspended in 1M PBS solution (pH 8). UIJ was aliquoted in Hungate tubes (9ml per tube). Prior to the experiment, a freshly prepared solution of Pancrex (final concentration 1 g.L<sup>-1</sup>, Paines and Byrne Ltd, UK) was added to Hungate tubes and incubated

anaerobically at 37°C for 5 min. One millilitre of overnight culture was subsequently inoculated. At T 0 and 2 hours, one millilitre sample was taken and diluted serially eight times

in half-strength peptone as described previously. Dilutions 1 to 8 were plated out on MRS

agar in triplicates. Plates were incubated for 48h in anaerobic cabinet at 37°C before enumeration.

#### Survival in simulated large intestine

A validated model of the human colon reflecting the physico-chemical conditions in the proximal, transverse and distal regions of the colon was used to determine the survival of probiotic strains in mixed culture environment (Macfarlane et al., 1998).

#### Gut model system

The system consists in three vessels V1, V2 and V3 arranged in series, with respective working volume of 0.28 L, 0.30 L and 0.30 L. Temperature was maintained at 37°C and the pH was automatically controlled to maintain values of 5.5 (V1), 6.2 (V2) and 6.8 (V3). All 10

vessels were kept under a headspace of oxygen-free nitrogen gas (0.9 L.h<sup>-1</sup>) and continuously stirred. The culture medium feeding the system was reflecting the components

entering the proximal colon and was prepared according to validated protocol (Macfarlane et

al., 1998 ). The culture medium was kept sterile and anoxic by sparging O<sub>2</sub>-free N<sub>2</sub>.

#### Medium

was fed to V1 by a peristaltic pump which sequentially supplied V2 and V3. Flow rate was set

to obtain a final retention time of 36 hours throughout the model.

#### Preparation of inoculum

Vessels were sterilised and filled with autoclaved feeding medium via the peristaltic pump. To

maintain anaerobiosis oxygen-free nitrogen gas was sparged in the system for 12 hours prior

to inoculation with faecal microflora. Inoculum consisted in 100ml of faecal slurry from a healthy human donor. The slurry was prepared by homogenizing freshly collected faeces in anoxic PBS at pH 7.2 (final concentration 200 g.L<sup>-1</sup>). The cultures were allowed to equilibrate

overnight before fresh medium was added to the system.

#### Experimental design and sampling protocol

Gut Model was run until equilibration was reached. Stability period was determined when seven turnovers of the feeding medium have run through the complete system. When steady

state conditions were reached, a total of 4 ml of a washed probiotic preparation was added daily to V1 for a period of five consecutive day (Day 1 to Day 5). From Day 6 to day 10, the system was run with no probiotic addition to measure the survival of probiotic in the mixed

culture environment. This experimental design was adopted to simulate the daily human consumption of probiotic product. Five millilitres of samples were taken from each vessel daily

for analysis of bacterial composition.

#### Preparation of probiotic strain for inoculation

To differentiate the probiotic strain fed to the multi-stage gut model from similar bacterial

species present in the indigenous microflora, rifampicin-resistant mutant of the studied strains

were isolated. The resistant variant of probiotic were selected by growing successive overnight, anaerobic cultures in MRS broth containing increasing amount of rifampicin (0.100

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to 100  $\mu\text{g.L}^{-1}$ ) until growth was observed in the medium with the highest antibiotic concentration. Rifampicin-resistant strains were isolated on MRS agars supplemented with rifampicin (100  $\mu\text{g.L}^{-1}$ , sigma, UK) and kept on cryogenic beads in Microbank tubes at -70°C.

Prior to the study, overnight cultures (100ml) of the probiotic strain were grown in MRS broth

supplemented with rifampicin (100  $\mu\text{g.L}^{-1}$ ) under anaerobic condition. Cells were centrifuged

at 3000 rpm (5 min) and washed in 20 ml 1M PBS (pH 7.2) twice. The final pellet was re-suspended in 5 ml of feeding medium. One millilitre of concentrated probiotic was kept for numeration while the remaining 4 ml were added to V1.

Numeration of probiotic cells in the gut model

The concentration of probiotic inoculated to the model as well as the survival of probiotic in

vessels V1, V2 and V3 was numerated on MRS agar supplemented with rifampicin (100  $\mu\text{g.L}^{-1}$ )

1) after serial dilution in anoxic half strength peptone water. Dilution 1 to 8 were plated in duplicate and anaerobically incubated for 72h prior to numeration.

Analysis of bacterial composition

Fluorescent in situ hybridisation was used to numerate the predominant gut genera present in

the gut model (Harmsen et al, 1999). Genus specific 16S rRNA-targeted oligonucleotide probes labelled with the fluorescent dye Cy 3 were used for enumerating Bifidobacterium, Bacteroides, Lactobacillus/Enterococcus and Clostridium subgroup histolyticum bacteria. Samples (375  $\mu\text{l}$ ) were fixed with filtered paraformaldehyde (1125 $\mu\text{l}$  of a 40  $\text{g.L}^{-1}$  solution). The remaining procedure was identical to that described by Rycroft et al, 2001. Total number

of bacterial cells was assessed using the nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis

Statistical analysis was performed using two-tailed Student's t-test, assuming equal variance

between two samples. Statistical significance was  $p < 0.05$ .

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Results and discussion

Prior to the commencement of this study, a total of thirty five bacterial strains were isolated

and identified from commercial products. The identity of the bacterial strains and their subsequent use in the series of tests is summarised in Table 1. Strains included 19 isolates of

Lactobacillus spp., 5 isolates of Bifidobacterium spp. One isolate of Lactococcus lactis sp. and 5 isolates of Enterococcus sp. The sources of each of these probiotic strains were from products available in UK supermarkets and health stores as summarised in Table 2.

Commercial products were chosen to be representative of the formulation currently available

on the market. These comprised dairy products, yoghurts and fruit juices containing live bacteria or dry preparations in the form of capsule, tablets or powder.

Survival in simulated stomach contents

The typical transit time of a small food bolus in the stomach is approximately 20 minutes.

This

period of time was therefore chosen to test the resistance of bacterial strains to simulated gastric contents. Stomach acidity varies according to individuals and whether individuals has

fasted prior to ingestion. To account for these inter-individual differences, the ability of probiotic strains to survive at various acidic pHs was investigated. The effects of simulated gastric digestion on 32 strains from our collection are presented in Table 3. Lactobacillus casei (isolate BeIi2), Lactobacillus plantarum (isolate BeH1), Enterococcus faecium (isolate RbC2) could not be revived adequately on WC agar and were not included in the selection.

At

pH 1, 17 strains did not survive the stringent acidity of the simulated gastric medium immediately after inoculation as shown by the low recovery of cells at T0 for isolates BeC1, BeC2, BeG1, BeIi1, RbC1, RbIi1, RbIi2, Strain 46, Strain 47, WCA1, WCA3, WCC1, WCD1, WCF1, WCG2, WCG3, WCH1. Acidic stress may have repress cell growth and may explain the absence of colonies on agar plates. Some strains were able to recover after 20 min such as Lactobacillus pentosus WCA2. Stress due to pH acidity and subsequent recovery effects were observed to a lesser extent at pH 2 and 3. These events highlighted the variability in survival rate that has been reported in vivo. Strains were assessed according to their survival

rate as determined by the ratio of live cells T20 to T0. Among the strains most able to survive

gastric acid conditions, eight strains showed more than 50% survival at pH 2 and 3. They

13 were Bifidobacterium sp. (RbG1), L. acidophilus strain JVT5 (RgC2), L. reuterii (strain 48), L.

pentosus (WCA2), E. faecium (WCB1), L. delbrueckii subsp. bulgaricus strain JHWW5 (WCF1), L. plantarum (WCG1), L. acidophilus subsp johnsonii (WCG2). Additionally, L. acidophilus strain JVT5 (BeA1) and L. plantarum (RgH1 and WCH1) were amongst the most

resistant strains at pH 2. Finally seven strains displayed good survival at pH 3. They were Bifidobacterium longum (RbIi2), L. pentosus (RgA1), L. casei immunitass (strain 46), L. casei

shirota (strain 47), L. delbrueckii subsp. bulgaricus strain JHWW5 (WCA3), Lactococcus lactis

subsp. lactis IL1403 (WCC1) and Bifidobacterium lactis (WCC2). Strains resistant to pH 2 or 3

were likely to reach the upper intestine intact, they were thus selected for subsequent

experiments on bile acid tolerance and resistance to upper intestine environment.

#### Bile acid tolerance

The tolerance to bile acid was determined in increasing concentration of bile salts according

to Walker and Gililand's procedure (1993). Data obtained are summarised in Table 4.

*Lactobacillus plantarum* (BeH1, WCG1 and WCH1) and *L. pentosus* (RGA1 and WCA2) were the most tolerant strains in the tested conditions. Larger differences were observed between

different isolates of the same strain such as *L. delbrueckii* subsp *bulgaricus* (WCF1 and WCA3) and *L. acidophilus* strain JVT5 (BeA1 and RGC2). Generally the coefficient of inhibition was significantly lower at the highest concentration of bile, illustrating a negative effect of bile secretion in the upper intestine on the survival of probiotic cells.

#### Bifidobacterium

*lactis* (WCC2) and *Lactococcus lactis* subsp. *lactis* IL1403 showed poor growth in the control

treatment which made the assessment of tolerance to bile acids difficult for these specific strains. MRS substrate and anaerobic conditions used in this experiment may not be suitable

for assessment of *Bifidobacterium* spp. and *Lactococcus* spp.

#### Survival in upper intestine content

A method developed by Charteris et al. (1998) was used to determine the survival of probiotics in the presence of bile salts, pancreatic enzymes and alkaline pH. The effect of simulated intestine contents on probiotic strains is summarised in Table 5. *Enterococcus faecium* (RgC1), *Bifidobacterium lactis* (RbC1 and WCC2) were the least sensitive strains to

14 simulated upper intestine contents. *Lactococcus lactis* subsp *lactis* also showed good survival

in this test. The most sensitive probiotic strains were from *L. casei* and *L. acidophilus* group.

Results contrasted from those obtained with Walker and Gililand's test. In the present experiment, facultative anaerobic bacteria displayed better survival than organism requiring

anaerobiosis for optimal growth. The alkaline conditions (pH 8) may have also compromised

the survival of the most acidophilic bacteria present in our collection. Greater sensitivity of *Lactobacilli* spp. to simulated intestine content have been reported in other studies (Charteris

et al. 1998). The presence of milk protein or mucin was shown to increase survival of probiotic

in the upper intestine contents (Fernandez et al, 2003; Charteris et al, 1998). In our study, most of the probiotic strains were presented to the consumers in the form of dairy products or

enteric coated capsules. Milk protein and enteric coating may limit the deleterious effect of upper intestine contents when these product are ingested.

#### Survival in simulated large bowel content

Survival in the large bowel content was assessed for *Lactobacilli* showing the strongest acid

and bile tolerance. Seven *Lactobacillus* strains were selected: *L. casei* immunitass (strain 46), *L. casei* shirota (strain 47), *L. plantarum* (WCH1), *L. pentosus* (WCA2), *L. reuterii* (strain 48), *L. acidophilus* subsp. *johnsonii* (WCG2) and *L. delbrueckii* subsp. *bulgaricus* (WCF1). Rifampicin-resistant mutants were obtained to discriminate probiotic strains from similar species present in the residential microflora of the three-stage continuous system.

Rifampicin resistance was obtained by chromosomal genetic alteration thereby preventing distribution of the rifampicin-resistance among the residential microflora. Concentrations of probiotic strain present in each compartment of the three-stage system are summarised in Table 6. In the inoculum, probiotic concentrations showed variability between strains. Inocula from *Lactobacillus plantarum* and *Lactobacillus pentosus* contained a concentration of live cells in the range of  $10^{10}$  CFU/ml whereas inocula from *Lactobacillus reuterii*, *Lactobacillus acidophilus* subsp. *johnsonii* and *Lactobacillus delbrueckii* subsp. *bulgaricus* contained on average  $10^5$  to  $10^6$  CFU/ml. *Lactobacillus immunitass* was fastidious to grow in the chosen conditions with no live cell surviving in the inoculum at day 2, 4 and 5. Ideally a yield of  $10^7$  CFU/ml was expected and seed cultures were incubated until corresponding absorbance reached 1.5. A greater sensitivity to the centrifugation and concentration procedure may have led to the low survival observed for *Lactobacillus acidophilus* subsp. *johnsonii*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus immunitass*.

During the inoculation period (Day 1 to Day 5), probiotics were found in higher concentrations in Vessel 1. The acidic conditions and availability of substrates in vessel 1 may explain a higher level of colonisation of this vessel. However *L. reuterii* colonised primarily vessel 2 whereas *L. acidophilus* subsp. *johnsonii* was present at its highest level in vessel 3. When probiotic inoculations were stopped, colonisation profile changed for all probiotic strains although no statistical difference was found between the concentrations of cells present during the inoculation period and the wash-out period in all vessels (Table 6). Due to poor numbers of cells in the inoculum, *L. casei* immunitass did not survive in the three-stage fermentation system (Figure 1). *L. shirota* was established at its higher level in vessel 2 at Day 6 (Figure 2). When the addition of fresh inoculum was stopped (Day 5), a decrease of the population levels were observed in vessel 2 and 3. Level of colonisation in vessel 1 remained stable at  $10^6$  cells/ml throughout the wash-out period suggesting that *L. casei* shirota could potentially survive and compete with the residential gut microflora. *L. plantarum* successfully colonised the three vessels of the fermentation system with the highest concentration in

vessel 1. After five days of inoculation, the strain appeared to be persisting in the gut model and able to maintain a substantial population in the range of  $10^6$  cells/ml (Figure 3). Of all the

strains studied, *L. plantarum* showed the highest colonisation in the three stage fermentation

system. *Lactobacillus pentosus* demonstrated a similar profile to *L. plantarum*, with a rapid colonisation of the three fermentation vessels (Figure 4). The concentration was the highest in

vessel 1 at Day 4. From Day 5 *L. pentosus* gradually decreased to a residual level of  $10^4$  cells/ml. *L. reuterii* was able to survive in the gut model system but the level of colonisation was lower than *L. plantarum* and *L. pentosus* (Figure 5). Daily variations were found in the number of probiotic cells recovered in vessel 1. *L. reuterii* showed preferential growth in vessel 2 and 3 but strain survival declined in the last days of the wash-out periods (Day 8, 9 and 10) suggesting a transient colonisation of *L. reuterii* in the large bowel when the strain was not fed continuously. *L. acidophilus* subsp. *johnsonii* showed a delayed colonisation of the fermentation vessels due to the low concentrations of cells in the inocula. (Figure 6).

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Vessel 2 had on average the highest colonisation. A residual number of probiotic cells were persisting at day 10. A delay in colonisation was also observed for *L. delbrueckii* subsp. *bulgaricus* (Figure 7). Although the strain was present at lower level than other probiotic strain

in the inoculation period, the numbers of cells increased over the wash out period to reach a

final concentration greater than those observed for *L. reuterii* and *L. acidophilus* subsp. *johnsonii*.

Overall *L. plantarum* showed the best ability to colonise the three compartment of the lower

digestive tract for the period of the study followed by *L. pentosus*, *L. casei shirota* and *L. reuterii*. The survival of *L. casei shirota* and *L. reuterii* have already been documented in vivo

(Yuki et al., 1999; Casas et al., 2000). Generally strains coming from human sources such as *L. plantarum* and *L. reuterii* were most able to survive in the lower digestive tract than dairy

strains such as *L. acidophilus* subsp. *johnsonii* and *L. delbrueckii* subsp. *bulgaricus*. A preferential colonisation of Vessel 1 may be explained by the large availability of carbohydrates for fermentation. The maintenance of an acidic pH may also be beneficial for Lactobacilli strains. The ability of probiotic strains to survive in Vessel 2 and 3 where the amount of carbohydrates is reduced is beneficial for the host. These probiotic bacteria may maintain fermentative activities in the distal part of the gut thereby preventing the release of

deleterious breakdown products from bacterial proteolytic activities.

Influence of probiotics on the composition of the gut microflora

Total number of cells in vessel 1, 2 and 3 was in the range of  $10^9$  bacteria /ml of gut model medium (Table 7) which is in good correlation to levels found in the bowel in vivo (Macfarlane

et al., 1998). Overall, little variation of the total number of cells present in the three different vessels were observed between Day 1 and Day 10, although a substantial number of probiotic bacteria were added daily. This is in accordance with the bacterial homeostasis found in the gut. In this experiment, four major groups of intestinal bacteria were quantified to determine whether probiotics influenced the microbial composition of the indigenous microflora. Bacteroides were numerically predominant populations and numbers were generally inversely correlated with the presence of bifidobacteria. There was a trend to a decrease of bifidobacterial group between Day 1 and Day 10. The addition of probiotic from the lactobacilli group did not contribute to the variation in this population.

17

18  
Conclusions

- Summary of main findings

The project has generated a whole data set on the ability of commercial strains to survive in

vitro conditions that mimic individual physiological environments of the intestinal tract.

The

choice of in vitro systems allowed direct comparison between bacterial strains. A

procedure

difficult to achieve in in vivo experiments. The collection of strains used in this project covers

most of the probiotic bacteria referenced in the literature and therefore can also be relevant to

other commercial products that were not tested during the course of the project. Lower intestinal challenge data are not currently available in the literature. These data constitute important information and material for publication.

- How closely objectives were met

Many probiotic products are available and our data indicates that the products that are most

known from the consumer were found to match their content in quality and content. Not all bacterial strains used as probiotics have the ability to survive the intestinal digestion.

Overall

the study showed that *Lactobacillus* spp. were resistant to the gastric environment but were

sensitive to upper intestinal content. Conversely, *Bifidobacterium* spp. were more likely to be

affected by stomach digesta but survived well in the upper intestine compartment. The project

enabled the consolidation of research methodologies available for determination of probiotic

functionality. This approach brought information on probiotic survival limitations and survival

in the gut.

- Limitations in the approach taken

Probiotic strains were studied individually in standardised conditions that may not have been optimum for their specific growth. Commercial products are diverse and some may contain a mixture of several strains. Interaction between probiotic strains may enhance survival and the biological effect. For instance probiotic VSL#3 containing high concentrations of several probiotic strains was shown to enhance the colonisation of the intestinal environment by ingested probiotic bacteria (Brigidi et al., 2003). Similarly the presence of milk protein and specific substrates such as lactose or other oligosaccharides in commercial probiotic products may optimise the survival of probiotic strains. The successful improvement of probiotic survival in synbiotic products – products combining probiotic strains and resistant carbohydrates - has been demonstrated for *Lactobacillus* and *Bifidobacterium* spp. (De Boever et al, 2001; Desmond et al, 2002; Crittenden et al., 2001; Wang et al., 1999) Moreover, the intake of a food bolus at the time of probiotic ingestion may also affect the survival of probiotic bacteria. These changes were accounted for to a limited level in the gastric and upper intestine contents simulation where varying pHs and bile concentrations were tested. In our approach however, bacteria were sub-cultured on preferential growth agar prior to testing. The integrated course of event from storage to passage in the different gut compartments was not represented fully in this study and would need further investigation. Finally, survival of probiotic strain through the digestive tract does not systematically lead to beneficial response from the host. The combination of digestive bacterial homeostasis and mature immune system in healthy subjects may mask the potential probiotic effect. The effect may however be effective in populations where the immune system is immature or weak such as infants or the elderly. A thorough in vivo investigation of the probiotic strains included in this study as well as more information on the impact of probiotics on the immune system would be required to achieve a comprehensive overview of the efficacy of probiotics in human.

Macronutrient metabolism by the human gut microbiome: major fermentation byproducts and their impact on host health Kaitlyn Oliphant\* and Emma Allen-Vercoe  
 Abstract The human gut microbiome is a critical component of digestion, breaking down complex carbohydrates, proteins, and to a lesser extent fats that reach the lower gastrointestinal tract. This process results in a multitude of microbial metabolites that can act both locally and systemically (after being absorbed into the bloodstream). The impact of these biochemicals on human health is complex, as both potentially beneficial and

potentially toxic metabolites can be yielded from such microbial pathways, and in some cases, these effects are dependent upon the metabolite concentration or organ locality. The aim of this review is to summarize our current knowledge of how macronutrient metabolism by the gut microbiome influences human health. Metabolites to be discussed include short-chain fatty acids and alcohols (mainly yielded from monosaccharides); ammonia, branched-chain fatty acids, amines, sulfur compounds, phenols, and indoles (derived from amino acids); glycerol and choline derivatives (obtained from the breakdown of lipids); and tertiary cycling of carbon dioxide and hydrogen. Key microbial taxa and related disease states will be referred to in each case, and knowledge gaps that could contribute to our understanding of overall human wellness will be identified.

Keywords: Human gut microbiome, Microbial metabolism, Macronutrients, Human health

Introduction The human gut microbiota is a complex ecosystem of microorganisms that inhabits and critically maintains homeostasis of the gastrointestinal (GI) tract [1]. Most of the contributions made by the gut microbiota to the physiology of the human superorganism are related to microbial metabolism [2–4], with bacteria being the largest of these contributors to ecosystem functioning in terms of relative genetic content [2]. In general, microbial metabolism of both exogenous and endogenous substrates to nutrients useable by the host is the direct benefit, but metabolites can also act to modulate the immune system through impacting the physiology and gene expression of host cells [3, 5, 6]. The colon is the major site of this fermentation, as its relatively high transit time and pH coupled with low cell turnover and redox potential presents more favorable conditions for the proliferation of bacteria [7]. However, that does not preclude the importance of the microbiota at other sites, as for example, the small intestinal microbiota has been shown to regulate nutrient absorption and metabolism conducted by the host [8]. Further, the presence of diverse metabolic activity can allow the microbiota to maximally fill the available ecological niches and competitively inhibit colonization by pathogens at all sites [9–11]. The elevated concentrations of the mostly acidic fermentation by-products also locally reduce the pH to create a more inhospitable environment for these incoming invaders [11]. However, specific fermentation pathways carried out by gut microbes can result in the formation of toxic compounds that have the potential to damage the host epithelium and cause inflammation [12–14]. The three macronutrients consumed in the human diet, carbohydrates, proteins, and fat, can reach the colon upon either escaping primary digestion once the amount consumed exceeds the rate of digestion, or resisting primary digestion altogether due to the inherent structural complexity of specific biomolecules [14–16]. Several factors can influence digestive efficiency, which in turn modulates the substrates available to the gut microbiota for consumption, including the form and size of the food particles (affected by cooking and processing), the composition of

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the meal (affected by the relative ratios of macronutrients and presence of anti-nutrients such as  $\alpha$ -amylase inhibitors), and transit time [17]. Transit time in particular has been shown to increase the richness and alter the composition of fecal microbial communities [18], which itself results from several variables including diet, physical activity, genetics, drugs (e.g., caffeine and alcohol), and psychological status [19]. The bioavailability of micronutrients to the host can also be influenced by gut microbial metabolic processes. Colonic bacteria can endogenously synthesize essential co-factors for host energy metabolism and regulation of gene expression, such as B vitamins [20]. Another example includes the biotransformation of exogenous plant-derived polyphenols that have anti-oxidant, anticancer, and/or anti-inflammatory properties by the gut microbiota, which improves their uptake by the host [21]. The following review articles on micronutrients are recommended to readers since this topic encompasses a wide scope of material [20, 21], as such, the predominant food sources that act as precursors for the most highly concentrated metabolites will be the focus of discussion here. The aim of this review is thus to describe the major microbial fermentation by-products derived from macronutrients and their subsequent impacts on host health.

**Primary degradation** Dietary polysaccharides can be interlinked in complex ways through a diverse array of bonds between monosaccharide units, reflected by the sheer number of carbohydrate-activating enzymes reported to have been found in the human gut microbiome [22]. For example, *Bacteroides thetaiotaomicron* possesses 260 glycoside hydrolases in its genome alone [23], which emphasizes the evolutionary requirement for adaptation in order to maximize utilization of resistant starch and the assortment of fibers available as part of the human diet. In contrast, human cells produce very few of these enzymes (although they do produce amylase to remove  $\alpha$ -linked sugar units from starch and can use sugars such as glucose, fructose, sucrose, and lactose in the small intestine) and so rely on gut microbes to harvest energy from the remaining complex carbohydrates [17, 24]. However, once the rate-limiting step of primary degradation is surpassed, the resulting monosaccharides can be rapidly consumed by the gut microbiota with often little interconversion necessary for substrates to enter the Embden-Meyerhof-Parnas pathway, Entner-Doudoroff pathway, or Pentose phosphate pathway for pyruvate and subsequent ATP production [25]. Conversely, dietary proteins are characterized by conserved peptide bonds that can be broken down by proteases; gut bacteria can produce aspartic-, cysteine-, serine-, and metallo-proteases, but in a typical fecal sample, these bacterial enzymes are far outnumbered by proteases arising from human cells [26]. However, the 20 proteinogenic amino acid building blocks

require more interconversion steps for incorporation into biochemical pathways in comparison to monosaccharide units, and thus it is not typical for a given gut microbial species to have the capacity to ferment all amino acids to produce energy [27]. Additionally, microbial incorporation of amino acids from the environment into anabolic processes would conserve more energy in comparison to their catabolic use, by relieving the necessity for amino acid biosynthesis [13]. It is for this reason that amino acids are generally not considered to be as efficient of an energy source as carbohydrates for human



Acetate Carbon dioxide and Hydrogen Formate Lactate Methanol Proteins Saccharides  
 Acetogenesis Acetate production Butyryl c CoA transferase pathway Ethanol production  
 Lactate production Acetate Butyrate Carbon dioxide and Hydrogen Ethanol Formate  
 Lactate Erysipelotrichaceae Erysipelatoclostridium Proteins Saccharides Acetate  
 production Lactate production Acetate Carbon dioxide and Hydrogen Formate Lactate  
 Lachnospiraceae Blautia (Clostridium cluster XIVa) 1,2-Propanediol Carbon dioxide and  
 Hydrogen Dietary carbohydrates Formate Mucin 1,2-Propanediol pathway Acetogenesis  
 Acetate production Ethanol production Lactate production Succinate pathway I Acetate  
 Carbon dioxide and Hydrogen Ethanol Formate Lactate Propanol Propionate  
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Table 1 Major genera present in the human gut microbiome and their metabolisms  
 (Continued) Phylum Family Genus Substrates Metabolism End products Succinate  
 Coprococcus (Clostridium cluster XIVa) Acetate Dietary carbohydrates Lactate Acrylate  
 pathway Butyrate kinase pathway Butyryl CoA:acetyl CoA transferase pathway Ethanol  
 production Lactate production Acetate Butyrate Ethanol Carbon dioxide and Hydrogen  
 Formate Lactate Propionate Dorea (Clostridium cluster XIVa) Dietary carbohydrates  
 Acetate production Ethanol production Lactate production Acetate Carbon dioxide and  
 Hydrogen Ethanol Formate Lactate Lachnoclostridium (Clostridium cluster XIVa) Proteins  
 Saccharides Acetate production Butyrate kinase pathway Ethanol production Lactate  
 production Acetate Butyrate Carbon dioxide and Hydrogen Ethanol Formate Lactate  
 Roseburia (Clostridium cluster XIVa) 1,2-Propanediol Acetate Dietary carbohydrates 1,2-  
 Propanediol pathway Acetate production Butyryl CoA:acetyl CoA transferase pathway  
 Ethanol production Lactate production Acetate Butyrate Carbon dioxide and Hydrogen  
 Ethanol Formate Lactate Propanol Propionate Lactobacillaceae Lactobacillus 1,2-  
 Propanediol Saccharides 1,2-Propanediol pathway Acetate production Ethanol production  
 Lactate production Acetate Ethanol Formate Lactate Propanol Propionate  
 Ruminococcaceae Faecalibacterium (Clostridium cluster IV) Acetate Butyryl CoA:acetyl  
 CoA transferase pathway Butyrate Carbon dioxide and Hydrogen Formate  
 RuminiclostridiumW (Specifically Clostridium cluster IV, which is currently grouped with  
 Clostridium cluster III) Dietary carbohydrates Proteins Acetate production Butyrate kinase  
 pathway Ethanol production Lactate production Acetate Butyrate Carbon dioxide and  
 Hydrogen Ethanol Formate Lactate Ruminococcus (Clostridium cluster IV) Dietary  
 carbohydrates Acetate production Ethanol production Lactate production Succinate  
 pathway I Acetate Ethanol Formate Lactate Succinate Streptococcaceae StreptococcusNW  
 Mucin Saccharides Acetate production Ethanol production Lactate production Acetate  
 Ethanol Formate Lactate

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not metabolized by IECs are transported via the hepatic vein to the liver, where they can be incorporated as precursors into gluconeogenesis, lipogenesis, and cholesterogenesis [62]. Specifically, propionate is gluconeogenic, whereas acetate and butyrate are lipogenic. The ratio of propionate to acetate is thought to be particularly important, as propionate can inhibit the conversion of acetate to cholesterol and fat [62, 66]. Indeed, propionate administration alone can reduce intra-abdominal tissue accretion and intrahepatocellular lipid content in overweight adults [67]. The role(s) of SCFAs in glucose homeostasis is/are not yet fully elucidated, although preliminary work has additionally suggested a beneficial effect, since plasma insulin levels are inversely related to serum acetate concentrations [62,

68]. In addition to SCFAs, small but significant amounts of alcohols, including ethanol, propanol, and 2,3-butanediol, can be formed as end-products of pyruvate fermentation (Table 1; Fig.1). A further alcohol, methanol, is also produced by the gut microbiota as a result of pectin degradation, demethylation of endogenous cellular proteins for regulation, or vitamin B12 synthesis [69] rather than fermentation. Alcohols are transported to the liver, where the detoxification process involves their conversion to SCFAs, although through pathways that yield toxic aldehydes as precursors [69–71]. Higher concentrations of endogenous alcohols are thus thought to be a contributing factor to the development of non-alcoholic fatty liver disease (NAFLD) [70, 72]. Proteobacteria are known to be particularly capable of alcohol generation [69, 72], and are, interestingly, positively associated with dysbiosis in inflammatory bowel disease (IBD) [73], a disease in which patients are predisposed to developing NAFLD [74]. However, alcohols can also be detoxified by many members of the gut microbiota via pathways similar to those present in mammalian cells, regulating their concentration [69]. Additionally, methanol can be used as a substrate for methanogenesis or acetogenesis [35, 69, 75], and ethanol can be coupled to propionate for fermentation to the SCFA, valerate (Table 1)[36]. Valerate is a poorly studied metabolite, but it has been shown to inhibit growth of cancerous cells [76] and to prevent vegetative growth of *Clostridioides difficile* both in vitro and in vivo [36].

**Hydrogenotrophy** The human body may rapidly absorb SCFAs and alcohols, which helps to reduce their nascent concentrations within the colon, allowing for continued favorable reaction kinetics [15, 77]. In addition, the gaseous fermentation byproducts, carbon dioxide and hydrogen, must also be removed to help drive metabolism forward. The utilization of these substrates is mainly the result of cross-feeding between gut microbiota members, rather than host absorption. Three main strategies for this activity exist in the human gut: (1) acetogens, for example, *Blautia* spp., convert carbon dioxide plus hydrogen to acetate (further examples included in Table 1); (2) methanogens, namely archaea

**Table 1 Major genera present in the human gut microbiome and their metabolisms (Continued)**

Phylum	Family	Genus	Substrates	Metabolism	End products	
	Veillonellaceae	<i>Veillonella</i>	1,2-Propanediol	Lactate	Proteins	
			Saccharides	Succinate	1,2-Propanediol pathway	
			Acetate production	Lactate production	Succinate pathway	
			Acetate	Carbon dioxide and Hydrogen	Formate	
			Lactate	Propanol	Propionate	
			Succinate	Proteobacteria		
	Enterobacteriaceae	<i>Escherichia</i>	Proteins	Saccharides	1,2-Propanediol pathway	
			1,2-Propanediol production	Acetate production	Ethanol production	
			Lactate production	Succinate pathway	1,2-Propanediol	
			2,3-Butanediol	Acetate	Carbon dioxide and Hydrogen	
			Ethanol	Formate	Lactate	
			Succinate	Taxa that are listed as part of a ‘core’ gut microbiota found by Falony et al. are in bold [31]. Those genera that were core components of exclusively the ‘Western’ cohorts are denoted with a ‘W’ superscript, whereas the exclusively ‘non-Western’ ones are denoted with a ‘NW’ superscript. If the core taxon could not be resolved to the genus level, the bacterial families are bolded. For the bacterial families that do not already contain several core genera, the most commonly described genus of the human gut microbiome for that family is also listed as a representative. Additionally, genera found to be highly prevalent among the human population, yet typically present in low abundance, are underlined [32]. The possible substrates consumed, metabolisms, and metabolites for each genus are listed. These metabolisms were inferred from the following articles [28, 33–61]. Note that many of these metabolisms are species-		

specific, and only the substrates commonly utilized among species of the genus are listed. Further, only the most abundant metabolites produced from pyruvate catabolism (i.e., saccharolytic processes) are given. When a particular metabolic pathway is denoted with an 'T' superscript, the microorganisms do not possess the full enzymatic pathway, but rather produce the typical intermediate as an end-product instead. Likewise, an '1/A' indicates species of that genus may possess either the full or half pathway

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such as *Methanobrevibacter*, convert carbon dioxide plus hydrogen to methane; and (3) sulfate reducing bacteria, including *Desulfovibrio*, convert sulfate plus hydrogen to hydrogen sulfide [15, 37]. A higher abundance of these cross-feeders may improve the overall efficiency of metabolism in the gut; for example, an increase in methanogens is observed in the GI tract of anorexia nervosa patients, which may be a coping strategy by the gut microbiota in response to a lack of food sources [78, 79]. Sulfate-reducing bacteria are the most efficient of the hydrogenotrophs, but require a source of sulfate; in the gut, the most prominent source of sulfate is sulfated glycans [80]. Although some of these glycans may be obtained from the diet, the most accessible source is mucin produced by the host [38]. Sulfate-reducing bacteria obtain sulfate from these substrates via cross-feeding with microbes such as *Bacteroides*, which produce sulfatases [80, 81]. Hydrogen sulfide is both directly toxic to IECs through inhibition of mitochondrial cytochrome C oxidase, and pro-inflammatory via activation of T helper 17 cells [82, 83]. Hydrogen sulfide can additionally directly act on disulfide bonds in mucin to further facilitate mucin degradation [84]. Elevated hydrogen sulfide concentrations and increased proportions of sulfate-reducing bacteria are reported in IBD [85].

Fig. 1 Strategies of pyruvate catabolism by the human gut microbiome. Carbohydrates are first degraded to pyruvate. Pyruvate may then be converted to succinate, lactate, acetyl CoA + formate/carbon dioxide + hydrogen, ethanol, or 2,3-butanediol. Succinate may, however, also be a direct product of carbohydrate fermentation. Succinate and lactate do not typically reach high concentrations in fecal samples, as they can be further catabolized to produce energy, but certain species do secrete them as their final fermentation end-product, which enables cross-feeding. Acetate is produced by two pathways; (1) through direct conversion of acetyl CoA for the generation of energy (brown) or (2) acetogenesis (red). Formate/carbon dioxide + hydrogen can also be substrates for methanogenesis. Propionate is produced by three pathways; (1) the succinate pathway (orange), (2) the acrylate pathway (green), or (3) the 1,2-propanediol pathway (blue). 1,2-Propanediol is synthesized from lactaldehyde or dihydroxyacetone phosphate, which both are products of deoxy sugar fermentation (e.g., fucose, rhamnose). Alternatively, lactaldehyde can be produced from lactate, or 1,2-propanediol can be fermented to propanol. Propionate can be coupled with ethanol for fermentation to valerate (gray). The precursor for butyrate, butyryl CoA, is generated from either acetyl CoA or succinate. Butyrate is then produced by two pathways; (1) the butyrate kinase pathway (pink) or (2) the butyryl CoA:acetyl CoA transferase pathway (purple). Butyrate-producing bacteria may also cross-feed on lactate, converting it back to pyruvate. Lactate may also be catabolized as part of sulfate reduction

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Catabolism of amino acids The digestibility of proteins by the host is more variable than that of carbohydrates and fats, and is influenced by the previously mentioned factors of food processing, macronutrient ratios, and transit time [14, 18], in addition to its source

(e.g., plant or animal), which also leads to different amino acid compositions available to the gut microbiota [14, 86]. The extra steps of interconversion required for amino acid fermentation yield a large diversity of by-products. Protein catabolism in the gut generally has a negative connotation, as compounds that are toxic to the host can result from this process, including amines, phenols/indoles, and sulfurous compounds [12–14]. However, it is important to note that not all amino acids are fermented to toxic products as a result of gut microbial activity; in fact, the most abundant end products are SCFAs [13, 14].

Therefore, it may not be protein catabolism per se that negatively impacts the host, but instead specific metabolisms or overall increased protein fermentation activity. It is thus important to examine these subtleties. A microbe can exhibit one of two strategies for the initial step of amino acid catabolism, either deamination to produce a carboxylic acid plus ammonia or decarboxylation to produce an amine plus carbon dioxide [12]. Ammonia can inhibit mitochondrial oxygen consumption and decrease SCFA catabolism by IECs, which has led to the assumption that excess ammonia production can negatively impact the host [87–89]. However, the gut microbiota also rapidly assimilates ammonia into microbial amino acid biosynthetic processes [13], and host IECs can additionally control ammonia concentration through conversion to citrulline and glutamine, or through slow release into the bloodstream [90, 91]. It is thus unclear how much protein catabolism is necessary to achieve toxic ammonia concentrations, and this may vary between hosts. This uncertainty, coupled with the multiple negative impacts amines can have on the host (discussed below), have led to speculation that deamination would improve host outcomes. Fortunately, deamination appears to be the more common strategy of amino acid catabolism by the gut microbiota, because high concentrations of SCFAs are produced from amino acid degradation via this pathway [12, 13]. The next steps depend on the class of amino acid starting substrate, with most eventually resulting in tricarboxylic acid cycle intermediates, pyruvate, or coenzyme A-linked SCFA precursors [39, 75]. An exception would be the series of Stickland reactions exhibited by certain Clostridia, in which a coupled oxidation and reduction of two amino acids occurs as an alternative to using hydrogen ions as the electron acceptor [40, 41]. Phosphate is simultaneously added to the reduced amino acid in this case, and thus oxidative phosphorylation for the production of ATP can occur directly from the resultant acyl phosphate. In turn, branched-chain fatty acids (BCFAs), such as isovalerate and isobutyrate, can be produced as end-products. Additionally, some gut microbial species, mainly from the class Bacilli, also possess a specialized branched-chain keto acid dehydrogenase complex to yield energy from the oxidized forms of the branched-chain amino acids directly, which also leads to BCFA production [13, 75]. The major SCFA and BCFA products generated from degradation of each amino acid are presented in Table 2. BCFAs are often used as a biomarker of protein catabolism, with the promoted goal to reduce their concentration in order to improve health outcomes [14]. However, little is actually known about the impact of BCFAs on host health. In fact, preliminary work has shown that BCFAs are able to modulate glucose and lipid metabolism in the liver similarly to SCFAs [93], and isobutyrate can be used as a fuel source by IECs when butyrate is scarce [94]. What is undisputed, however, are the negative consequences of the pro-inflammatory, cytotoxic, and neuroactive compounds yielded from the sulfur-containing, basic and aromatic amino acids.

Sulfur-containing amino acids Catabolism of the sulfur-containing amino acids, cysteine and methionine, results in the production of hydrogen sulfide and methanethiol,

respectively [13, 14], and a large number of taxonomically diverse bacterial species contain the requisite degradative enzymes within their genomes, including members of the Proteobacteria phylum, the Bacilli class, and the Clostridium and Bifidobacterium genera [13, 75]. Hydrogen sulfide can be methylated to methanethiol, which can be further methylated to dimethyl sulfide, and this methylation is thought to be part of the detoxification process due to the progressively less toxic nature of these compounds [95]. However, methanethiol may also be converted to hydrogen sulfide, then oxidized to sulfate, for detoxification; this sulfate can then be utilized by sulfate-reducing bacteria [80, 81, 95]. Indeed, this latter reaction has been observed in cecal tissue, and is part of the sulfur cycle of the gut [96]. The impact of hydrogen sulfide on host health has already been discussed, thus the focus will shift to the biogenic amines produced by basic amino acid fermentation and the phenol/indole compounds produced by aromatic amino acid fermentation. Basic amino acids A wide diversity of bacterial species within the gut microbiota can decarboxylate the basic amino acids, thus resulting in the formation of amine by-products shown in Additional file 1, including bifidobacteria, clostridia, lactobacilli, enterococci, streptococci, and members of the Enterobacteriaceae family [97]. The catabolism of arginine

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can produce agmatine by deamination, and/or putrescine, spermidine, and spermine as part of the polyamine synthesis pathway (Additional file 1). Agmatine inhibits the proliferation of IECs, which is thought to stem from its ability to reduce the synthesis and promote the degradation of other polyamines [98]. This effect may not be negative depending on the context; for example, the resultant decrease of fatty acid metabolism in tissues reduced both weight gain and the hormonal derangements associated with obesity in rats fed a high fat chow [99]. Agmatine also may be anti-inflammatory through inhibition of nitric oxide synthase [100], and is a candidate neurotransmitter, with agonism for  $\alpha 2$ -adenoceptors and imidazoline binding sites, while simultaneously blocking ligand-gated cation channels (NMDA class) [101]. The latter activity has therapeutic potential for remediating some forms of hyperalgesia and for its neuroprotectivity. Putrescine, on the other hand, is essential for the proliferation of IECs [102]. It is the precursor to spermidine/spermine, which are both able to relieve oxidative stress and promote cellular longevity through autophagy stimulation [103]. All three polyamines improve the integrity of the gut by increasing expression of tight junction proteins [104], promoting intestinal restitution [105] and increasing mucus secretion [105, 106]. Finally, both putrescine and spermine are able to inhibit the production of proinflammatory cytokines, such as IL-1, IL-6, and TNF- $\alpha$  [107, 108]. Therefore, any benefits of agmatine must be weighed against its consequent reduction of these polyamines; it may be effective in the treatment of certain conditions such as metabolic syndrome but could be detrimental in excess under normal conditions. Arginine can additionally be converted to glutamate, which can be deaminated to produce 4-aminobutyrate (GABA). GABA is the major inhibitory neurotransmitter of the central nervous system, and alterations in the expression of its receptor have been linked to the pathogenesis of depression and anxiety [109]. Administration of lactobacilli and bifidobacteria that produce GABA to mice and rats has resulted in a decrease of depressive behaviors, a reduction of corticosterone induced stress and anxiety, and lessened visceral pain sensation [109–111]. GABA can additionally regulate the proliferation of T cells and

thus has immunomodulatory properties [112]. Interestingly, chronic GI inflammation not only induces anxiety in mice, but depression and anxiety often present comorbidity with GI disorders, including irritable bowel syndrome (IBS) [109, 113]. The catabolism of histidine can produce histamine (Additional file 1). Histamine may be synonymous with its exertion of inflammation in allergic responses, but bacterially produced histamine has actually been shown to inhibit the production of the pro-inflammatory cytokines TNF- $\alpha$  in vivo [114], and IL-1, and IL-12 in vitro [115], while simultaneously preventing intestinal bacterial translocation. Histamine is also a neurotransmitter, modulating several processes such as wakefulness, motor control, dendritic cell activity, pain perception, and learning and memory [116]. Low levels of histamine are associated with Alzheimer's disease, convulsions, and seizures, and increasing its concentration has antinociceptive properties [117]. However, there is likely a range of suitable concentration, as high levels of histamine are associated with sleep disorders, Parkinson's disease, schizophrenia, and autism [116, 117]. The catabolism of lysine can produce cadaverine (Additional file 1). Cadaverine is a poorly studied metabolite; it can be toxic, but only in high amounts [13, 97]. Cadaverine has, however, been shown to potentiate histamine toxicity [118] and higher concentrations of cadaverine are associated with ulcerative colitis (UC) [119].

Table 2 Major products of amino acid fermentation by the human gut microbiota

Amino acid	Amino acid class	Major products
Aspartate	Acidic	Propionate
Glutamate	Acidic	Acetate, Butyrate
Alanine	Aliphatic	Acetate, Propionate, Butyrate
Glycine	Aliphatic	Acetate
Methylamine	Isoleucine	Aliphatic 2-Methylbutyrate or converted to Valine
Leucine	Aliphatic	Isovalerate
Proline	Aliphatic	Acetate
Valine	Aliphatic	Isobutyrate
Asparagine	Amidic	Converted to aspartate
Glutamine	Amidic	Converted to glutamate
Phenylalanine	Aromatic	Phenolic
SCFA	Phenylethylamine	Tryptophan
Aromatic	Indolic	SCFA
Tryptamine	Tyrosine	Aromatic 4-Hydroxyphenolic
SCFA	Tyramine	Arginine
Basic	Converted to other amino acids (mainly Ornithine)	Agmatine
Histidine	Basic	Acetate, Butyrate
Histamine	Lysine	Basic
Acetate, Butyrate	Cadaverine	Serine
Hydroxylic	Butyrate	Threonine
Hydroxylic	Acetate, Propionate, Butyrate	Cysteine
Sulfur-containing	Acetate, Butyrate,	Hydrogen sulfide
Methionine	Sulfur-containing	Propionate, Butyrate, Methanethiol

Listed are the compounds found to be above 1 mM concentration in in vitro fermentation experiments conducted by Smith and Macfarlane [92], in addition to the biogenic amines that can be produced by decarboxylation [12, 13]. Underlined are the products indicated as most abundant as reported in a review article by Fan et al. [12]

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Aromatic amino acids Aromatic amino acid degradation can yield a wide diversity of indolic and phenolic compounds that can act as toxins or neurotransmitters as shown in Additional file 2. The catabolism of tryptophan can produce tryptamine and indoles (Additional file 2). Tryptamine is a neurotransmitter that plays a role in regulating intestinal motility and immune function [120]. Particularly, it is able to interact with both indoleamine 2,3-dioxygenase and the aryl hydrocarbon receptor to heighten immune surveillance, and dampen the expression of pro-inflammatory cytokines, respectively [121, 122]. A lack of these activities has therefore been implicated in the pathology of IBD; although, it should be noted that most tryptophan metabolites can interact with these receptors, thus it is not tryptamine-specific [13, 120, 122]. Tryptamine can also both potentiate the inhibitory response of cells to serotonin and induce its release from enteroendocrine cells [120, 123]. Serotonin is a neurotransmitter involved in many

processes including mood, appetite, hemostasis, immunity, and bone development [13, 124]. Its dysregulation is thus reported in many disorders, including IBD [125], IBS [126], cardiovascular disease [127], and osteoporosis [128]. Tryptophan decarboxylation is a rare activity among species of the gut microbiota, but certain Firmicutes have been found to be capable of it, including the IBD-associated species, *Ruminococcus gnavus* [129, 130]. Indole, on the other hand, is a major bacterial metabolite of tryptophan, produced by many species of *Bacteroides* and *Enterobacteriaceae* [120]. It plays an important role in host defense, by interacting with the pregnane X receptor and the aryl hydrocarbon receptor [120]. This activity fortifies the intestinal barrier by increasing tight junction protein expression and downregulates the expression of proinflammatory cytokines [120, 131]. It also induces glucagon like peptide-1 (an incretin) secretion by enteroendocrine cells, inhibiting gastric secretion and motility, to promote satiety [132, 133]. Indole is additionally a signaling molecule for bacteria, influencing motility, biofilm formation, antibiotic resistance, and virulence, and shown to inhibit the colonization capabilities of pathogens such as *Salmonella enterica* [134]. However, indole overproduction can increase its export to the liver, where it is sulfated to indoxyl sulfate, a uremic toxin associated with chronic kidney disease [135]. Further, its effects as a signaling molecule for both enteroendocrine cells and bacteria are dose dependent, with high concentrations rendering it ineffective [120, 132, 134]. Other indole metabolites are additionally able to interact with the pregnane X receptor and/or aryl hydrocarbon receptor in a similar fashion, thus benefiting the host, but are less well studied [120]. The catabolism of tyrosine can produce tyramine, phenols, and p-coumarate (Additional file 2). Tyramine is a neurotransmitter that can be produced by certain gut bacteria via decarboxylation, including *Enterococcus* and *Enterobacteriaceae* [97]. It is infamous for causing the 'cheese reaction' hypertensive crisis in individuals taking monoamine inhibitor class drugs, although it can additionally cause migraines and hypertension in sensitive individuals or a mild rise in blood pressure when consumed in excess by the general populace [136]. Tyramine facilitates the release of norepinephrine that induces peripheral vasoconstriction, elevates blood glucose levels, and increases cardiac output and respiration [137]. It has also been shown to increase the synthesis of serotonin by enteroendocrine cells in the gut, elevating its release into circulation [124]. Phenol and p-cresol are phenolic metabolites that have been shown to both decrease the integrity of the gut epithelium and the viability of IECs [138, 139], and can be produced by many gut bacterial species, such as members of the *Enterobacteriaceae* and *Clostridium* clusters I, XI, and XIVa [140]. P-cresol in particular is genotoxic, elevates the production of superoxide, and inhibits proliferation of IECs [141]. P-cresol may additionally be sulfated to cresyl sulfate in the gut or liver, which has been found to suppress the T helper 1-mediated immune response in mice [142], and, interestingly, phenolic sulfation was found to be impaired in the gut mucosa of UC patients [143]. Indeed, the colonic damage induced by unconjugated phenols is similar to that observed in IBD [138]. Cresyl sulfate is also associated with chronic kidney disease, however, as it can damage renal tubular cells through induction of oxidative stress [144]. This compound is also particularly elevated in the urine of autistic patients, but a causative link in this case has not been elucidated [145]. The catabolism of phenylalanine can produce phenylethylamine and trans-cinnamic acid (Additional file 2). Unlike tyrosine and tryptophan, little is known about these phenylalanine-derived metabolites. Phenylethylamine is a neurotransmitter that functions

as an 'endogenous amphetamine' yielded from decarboxylation [136]. Through facilitating the release of catecholamine and serotonin, phenylethylamine in turn elevates mood, energy, and attention [146]. However, it has been reported that ingesting phenylethylamine can induce headache, dizziness, and discomfort in individuals with a reduced ability to convert it to phenylacetate, suggesting excessive amounts have negative consequences [136]. In terms of its production in the gut, phenylethylamine has thus been positively associated with Crohn's disease and negatively correlated with *Faecalibacterium prausnitzii* in one study [147]. The conversion of phenylalanine to trans-cinnamate and tyrosine to p-coumaric acid results in increased phenylpropionate and 4-hydroxyphenylpropionate concentrations, which in turn produce urinary metabolites associated with the 'chlorogenic acid' phenotype in rats, as suggested by Clayton [148]. These metabolic pathways were found to so far specifically occur within species of *Clostridium* and

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*Peptostreptococcus*, respectively [149, 150]. The chlorogenic acid phenotype is associated with both autism and schizophrenia, suggesting a role of altered aromatic amino acid metabolism in these disorders [148, 151, 152]. However, further research is still needed, as there remains no mechanistic explanation of these metabolites toward disease development. Further, both trans-cinnamic acid and p-coumaric acid are negatively associated with cardiovascular disease [153, 154]. P-coumaric acid, in particular, is a common phenolic compound derived from plant matter that has anti-inflammatory properties, and has been demonstrated to prevent platelet aggregation [155]. Thus, these metabolites may simply be an indicator of altered microbial metabolism in general, when found in excess.

**Catabolism of lipids** A very small proportion of total dietary fat reaches the colon (<5%) [16, 156]. Microorganisms in the gut are known to possess lipases, which can degrade triglycerides and phospholipids into their polar head groups and free lipids [16, 157]. Triglycerides represent 95% of total dietary fat, whereas phospholipids, mostly in the form of phosphatidylcholine, constitute a minor portion, but are also derived endogenously from bile acids [158]. Certain bacteria inhabiting the GI tract, including species of lactobacilli, enterococci, clostridia, and Proteobacteria, can utilize the backbone of triglycerides as an electron sink, reducing glycerol to 1,3-propanediol [159]. 3-Hydroxypropanal (reuterin) is an intermediate of this process that has been reported to accumulate extracellularly in cultures of *Lactobacillus* and *Enterococcus* spp. [160]. Reuterin has antimicrobial properties acting against pathogens and commensals alike [161], but it can also be spontaneously dehydrated to acrolein [71]. Acrolein is a highly reactive genotoxin, with an equivalent mutagenic potency to formaldehyde, raising concerns about this metabolic process [71, 159]. Meanwhile, choline can additionally be metabolized to trimethylamine by species of the gut microbiota, particularly Clostridia (especially members of *Clostridium* cluster XIVa and *Eubacterium* spp.) and Proteobacteria [162, 163]. Trimethylamine is oxidized in the liver to trimethylamine N-oxide [163, 164], which exacerbates atherosclerosis by promoting the formation of foam cells (lipid-laden macrophages) [164] and altering cholesterol transport [165]. High levels of serum trimethylamine N-oxide are thus associated with cardiovascular disease [166] and atherosclerosis [167]. However, it should be noted that active research in these areas is in its early stages, and thus the link between the gut microbiota-mediated lipid head group metabolism and health

consequences is still unclear. For example, a study on the metabolism of glycerol by fecal microbial communities found that only a subset could reduce it to 1,3-propanediol, and the authors did not detect any reuterin [159]. Further, some members of the gut microbiota (e.g., methylotrophs) can breakdown trimethylamine to dimethylamine, so the actual amount of trimethylamine that is available for transportation to the liver can be diverted, and this is likely to be influenced by interindividual variability in the composition of the gut microbiota [168]. In contrast to the polar head groups, microorganisms are not thought to have the ability to catabolize free lipids in the anaerobic environment of the gut [169]. However, free lipids have antimicrobial properties [169, 170] and can directly interact with host pattern recognition receptors. Particularly, saturated fatty acids are TLR4 agonists that promote inflammation [171], whereas omega-3 unsaturated fatty acids are TLR4 antagonists that prevent inflammation [172]. Interestingly, chronic inflammation co-occurring with obesity has been well described [173], and could be a result of the aforementioned pro-inflammatory properties of free lipids, the lack of anti-inflammatory SCFAs produced from carbohydrate fermentation (high-fat diets tend to be low in carbohydrates), or a combination of both. High-fat diets do have a reported impact on the composition of the gut microbiota, yet it is unclear whether it is the increased fat content per se or the relative decrease in carbohydrates, which often accompanies these diets, that is the chief influencer [16, 169]. Indeed, Morales et al. observed that a high-fat diet including fiber supplementation induces inflammation without altering the composition of the gut microbiota [16]. Regardless, the gut microbiota is required for the development of obesity, as shown in GF mice experiments, because of the ability of SCFAs to alter energy balance as previously discussed [174].

**Effect on endogenous substrate utilization** Metabolism of exogenous substrates greatly affects the use of endogenous substrates by the gut microbiota. Dietary fiber reduces the degradation of mucin, and the utilization of mucin is thought to cycle daily depending on the availability of food sources [175, 176]. Mucin is a sulfated glycoprotein [38], thus the same concepts of carbohydrate and protein degradation from dietary sources discussed above apply. However, it should be noted that mucin turnover by the gut microbiota is a naturally occurring process, and only when it occurs in elevated amounts does it have negative connotations. For example, *Akkermansia muciniphila* is a mucinutilizing specialist that is depleted in the GI tract of IBD [177] and metabolic syndrome [178] patients. *A. muciniphila* has a demonstrated ability to cross-talk with host cells, promoting an increase in concentration of glucagon-like peptides, 2-arabinoglycerol, and antimicrobial peptides that improve barrier function, reduce inflammation, and induce proliferation of IECs [179].

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Through this communication, *A. muciniphila* also, paradoxically, restored the thickness of the mucin layer in obese mice. Dietary fat intake can also alter the profile of bile acids. Dairy-derived saturated lipids increase the relative amount of taurine-conjugation, and this sulfurcontaining compound leads to the expansion of sulfatereducing bacteria in the gut [180]. Bile acid turnover is, however, a naturally occurring process, which modulates bile acid reabsorption, inflammation, triglyceride control, and glucose homeostasis from IEC signaling [181].

**Conclusions** The critical contributions of the gut microbiota toward human digestion have just begun to be elucidated. Particularly, more recent research is revealing how the impacts of microbial metabolism extend beyond the GI tract, denoting the so-called gut-brain (e.g.,

biogenic amines acting as neurotransmitters) [182], gut-liver (e.g., alcohols) [183], gut-kidney (e.g., uremic toxins such as cresyl sulfate) [135], and gut-heart (e.g., trimethylamine) [184] axes. The primary focus to date has been on the SCFAs derived mainly from complex carbohydrates, and crucial knowledge gaps still remain in this area, specifically on how the SCFAs modulate glucose metabolism and fat deposition upon reaching the liver. However, the degradation of proteins and fats are comparatively less well understood. Due to both the diversity of metabolites that can be yielded and the complexity of microbial pathways, which can act as a self-regulating system that removes toxic byproducts, it is not merely a matter of such processes effecting health positively or negatively, but rather how they are balanced. Further, the presentation of these substrates to the gut microbiota, as influenced by the relatively understudied host digestive processes occurring in the small intestine, is equally important. Future work could therefore aim to determine which of these pathways are upregulated and downregulated in disease states, such as autism and depression (gut-brain), NAFLD (gut-liver), chronic kidney disease (gut-kidney), and cardiovascular disease (gut-heart). Further, a combination of human- and culture- (in vitro and in vivo) based studies could resolve the spectrum of protein and fat degradation present among healthy individuals, in order to further our understanding of nutrient cycling in gut microbial ecosystems, and thus gain a necessary perspective for improving wellness.

Additional files

Additional file 1: Pathways of basic amino acid fermentation by the human gut microbiome. Pathways have been simplified to show major endproducts. Where 'SCFA' is listed, either acetate, propionate or butyrate can result from catabolism of the substrate. (PDF 181 kb)

Additional file 2: Pathways of aromatic amino acid fermentation by the human gut microbiome. Pathways have been simplified to show major end-products. Where 'SCFA' is listed, either acetate, propionate or butyrate can result from catabolism of the substrate. (PDF 174 kb)

Abbreviations APC: Antigen presenting cell; BCFA: Branched-chain fatty acid; GABA: 4Aminobutyrate; GI: Gastrointestinal; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; IEC: Intestinal epithelial cell; NAFLD: Nonalcoholic fatty liver disease; SCFA: Short-chain fatty acid; TLR: Toll-like receptor; UC: Ulcerative colitis

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Authors' contributions KO researched and wrote the manuscript. EA-V oversaw editing of the final version of the manuscript. All authors approved the final manuscript.

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Consent for publication Not applicable.

Competing interests EA-V is the co-founder and CSO of NuBiyota LLC, a company which is working to commercialize human gut-derived microbial communities for use in medical indications.

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**26. If you wanted to boost your immune system, what would you NOT want to do?**

- a. Take spore-based probiotics
- b. Sleep between 5-6 hours a night
- c. Take supplements of vitamins D<sub>3</sub> and K<sub>2</sub>
- d. Reduce emotional stress

### Vitamin D and the Immune System

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**Abstract** It is now clear that vitamin D has important roles in addition to its classic effects on calcium and bone homeostasis. As the vitamin D receptor is expressed on immune cells (B cells, T cells and antigen presenting cells) and these immunologic cells are all capable of synthesizing the active vitamin D metabolite, vitamin D has the capability of acting in an autocrine manner in a local immunologic milieu. Vitamin D can modulate the innate and adaptive immune responses. Deficiency in vitamin D is associated with increased autoimmunity as well as an increased susceptibility to infection. As immune cells in autoimmune diseases are responsive to the ameliorative effects of vitamin D, the beneficial effects of supplementing vitamin D deficient individuals with autoimmune disease may extend beyond the effects on bone and calcium homeostasis.

The immune system defends the body from foreign, invading organisms, promoting protective immunity while maintaining tolerance to self. The implications of vitamin D deficiency on the immune system have become clearer in recent years and in the context of vitamin D deficiency, there appears to be an increased susceptibility to infection and a diathesis, in a genetically susceptible host to autoimmunity.

The classical actions of vitamin D are to promote calcium homeostasis and to promote bone health. Vitamin D enhances absorption of calcium in the small intestine and stimulates osteoclast differentiation and calcium reabsorption of bone. Vitamin D additionally promotes mineralization of the collagen matrix in bone. In humans, vitamin D is obtained from the diet or it is synthesized in the skin (reviewed in [1]). As vitamin D is cutaneously produced after exposure to UV B light, its synthesis is influenced by latitude, season, use of sunblock and skin pigmentation. Melanin absorbs UVB radiation inhibiting the synthesis of vitamin D from 7-dihydrocholesterol. This initial vitamin D compound is inactive and it is next hydroxylated in the liver to form 25 OH vitamin D<sub>3</sub> (25 D). 25 D is also an inactive compound, but is the most reliable measurement of an individual's vitamin D status. It is converted in the kidney to the active compound 1,25 dihydroxy vitamin D (1,25 D) or calcitriol by 1- $\alpha$ -hydroxylase (CYP27B1), an enzyme which is stimulated by PTH. 1,25 D may be further metabolized to the inactive 1,24,25 vitamin D by 24-hydroxylase (CYP24). 1,25 D levels are tightly regulated in a negative feedback loop. 1,25 D both inhibits renal 1 $\alpha$ -hydroxylase and stimulates the 24-hydroxylase enzymes, thus maintaining circulating levels within limited boundaries and preventing excessive vitamin D activity/signaling.

Correspondence: Cynthia Aranow 350 Community Drive Manhasset, NY 11030 516 562-3837 516 562-2537 (fax) caranow@nshs.edu . Address for reprints: same as corresponding author This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process

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1,25 D acts on the intestine where it stimulates calcium reabsorption, and upon bone, where it promotes osteoblast differentiation and matrix calcification. The active hormone exerts its effects on these tissues by binding to the vitamin D receptor (VDR). This complex dimerizes with the retinoid X receptor (RXR) and the 1,25D-VDR-RXR heterodimer translocates to the nucleus where it binds vitamin D responsive elements (VDRE) in the promoter regions of vitamin D responsive genes and induces expression of these vitamin D responsive genes.

Many tissues other than the skeletal and intestine express the VDR including cells in the bone marrow, brain, colon, breast and malignant cells and immune cells suggesting that vitamin D may have functions other than calcium and bone homeostasis[2]. Additionally, tissues other than the kidney express 1- $\alpha$ -hydroxylase and are capable of converting 25 D to 1,25 D, in non-renal compartments[1, 3-4]. Therefore, in addition to its endocrine functions, vitamin D may act in a paracrine or autocrine manner. Some of the more recently recognized non-classical actions of vitamin D include effects upon cell proliferation and differentiation as well immunologic effects resulting in an ability to maintain tolerance and to promote protective immunity. As antigen presenting cells (macrophages and dendritic cells), T cells and B cells have the necessary machinery to synthesize and respond to 1,25 D, vitamin D may act in a paracrine or autocrine manner in an immune environment. Moreover, local levels of 1,25 D may differ from systemic, circulating levels as local regulation of the enzymes synthesizing and inactivating vitamin D are different from the controls originating in the kidney. The extrarenal 1- $\alpha$ -hydroxylase enzyme in macrophages differs from the renal hydroxylase as it is not regulated by PTH[5]. Instead, it is dependent upon circulating levels of 25 D or it may be induced by cytokines such as IFN- $\gamma$ , IL-1 or TNF- $\alpha$ [6]. Furthermore, the macrophage 24 hydroxylase enzyme is a non-functional splice variant, so there is no negative feedback of local 1,25 D production by 1,25 D.

Vitamin D and Protective Immunity  
Vitamin D has been used (unknowingly) to treat infections such as tuberculosis before the advent of effective antibiotics. Tuberculosis patients were sent to sanatoriums where treatment included exposure to sunlight which was thought to directly kill the tuberculosis. Cod liver oil, a rich source of vitamin D has also been employed as a treatment for tuberculosis as well as for general increased protection from infections[7].

There have been multiple cross-sectional studies associating lower levels of vitamin D with increased infection. One report studied almost 19,000 subjects between 1988 and 1994. Individuals with lower vitamin D levels (<30 ng/ml) were more likely to self-report a recent upper respiratory tract infection than those with sufficient levels, even after adjusting for variables including season, age, gender, body mass and race[8]. Vitamin D levels fluctuate over the year. Although rates of seasonal infections varied, and were lowest in the summer and highest in the winter, the association of lower serum vitamin D levels and infection held during each season. Another cross-sectional study of 800 military

recruits in Finland stratified men by serum vitamin D levels[9]. Those recruits with lower vitamin D levels lost significantly more days from active duty secondary to upper respiratory infections than recruits with higher vitamin D levels (above 40nmol). There have been a number of other cross-sectional studies looking at vitamin D levels and rates of influenza [10] as well as other infections including bacterial vaginosis[11] and HIV[12-13]. All have reported an association of lower vitamin D levels and increased rates of infection. Results of studies looking at potential benefits of administering vitamin D to decrease infection have not been consistent, most likely secondary to a number of methodologic concerns[14]. One recent well-designed prospective, double blind placebo study using an objective outcome, nasopharyngeal swab culture (and not self report), and a therapeutic dose

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of vitamin D showed that vitamin D administration resulted in a statistically significant (42%) decrease in the incidence of influenza infection[15].

The beneficial effects of vitamin D on protective immunity are due in part to its effects on the innate immune system. It is known that macrophages recognize lipopolysaccharide LPS, a surrogate for bacterial infection, through toll like receptors (TLR). Engagement of TLRs leads to a cascade of events that produce peptides with potent bactericidal activity such as cathelicidin and beta defensin 4[16]. These peptides colocalize within phagosomes with injected bacteria where they disrupt bacterial cell membranes and have potent antimicrobial activity [17].

Vitamin D plays an important part in the innate antimicrobial response. TLR binding leads to increased expression of both the 1- $\alpha$ -hydroxylase and the VDR[17-18]. This results in binding of the 1,25 D-VDR-RXR heterodimer to the VDREs of the genes for cathelicidin and beta defensin 4 and subsequent transcription of these proteins. Transcription of cathelicidin is absolutely dependent on sufficient 25 D[17]. It is now clear that transcription of beta defensin 4 requires binding of NFkB to appropriate response elements on the beta defensin 4 RNA[19]. TLR 2-1 signaling facilitates IL-1 receptor engagement which results in translocation of NFkB to its binding site[19].

Vitamin D and Autoimmune Disease There is increasing epidemiologic evidence linking vitamin D deficiency and autoimmune diseases including multiple sclerosis (MS), rheumatoid arthritis (RA), diabetes mellitus (DM), inflammatory bowel disease and systemic lupus erythematosus (SLE) (reviewed in reference[20]. Reports of low serum vitamin D predicting development of autoimmune disease in the future have been published for MS, autoimmune DM and RA[21-23]. There is also data linking decreased in utero exposure to vitamin D and islet cell autoimmunity[24]. Lower in utero exposure assessed by a lower maternal intake of vitamin D during pregnancy in women whose prospective child was at risk of developing autoimmune DM is associated with a statistically increased risk of the child developing pancreatic autoimmunity.

Vitamin D has also been shown to facilitate progression of existing autoimmune disease. In one study, 161 patients with an early undifferentiated connective tissue disease were followed for a mean of over 2 years[25]. Most patients did not progress and remained in an undifferentiated state. Thirty-five (21%) patients went on to develop a defined rheumatologic diagnosis including RA, SLE, Mixed Connective Tissue Disease, and Sjogren's

Disease while 126 did not progress. Baseline characteristics of the two groups were similar. Importantly, the mean vitamin D level was significantly lower in the group that progressed to a definitive disease.

There have been many studies of vitamin D status in lupus patients from across the globe (reviewed in [26]). Vitamin D levels are typically lower in patients than in disease or normal controls. Deficiency of vitamin D is extremely common, often with more than 50% of lupus patients with deficient levels and severe deficiency (vitamin D levels less than 10ng/ml) is not uncommon. Disease activity has been shown to correlate inversely with vitamin D in many but not all studies. Similar correlations between low levels of vitamin D and disease activity and severity have been observed in other autoimmune diseases such as MS and RA[27-30].

**Vitamin D and Immunologic Function** Vitamin D has numerous effects on cells within the immune system. It inhibits B cell proliferation and blocks B cell differentiation and immunoglobulin secretion[31-32].

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Vitamin D additionally suppresses T cell proliferation[33] and results in a shift from a Th1 to a Th2 phenotype[34-35]. Furthermore, it affects T cell maturation with a skewing away from the inflammatory Th17 phenotype[36-37] and facilitates the induction of T regulatory cells[38-41]. These effects result in decreased production of inflammatory cytokines (IL-17, IL-21) with increased production of anti-inflammatory cytokines such as IL-10 (Figure 1A). Vitamin D also has effects on monocytes and dendritic cells (DCs). It inhibits monocyte production of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12 and TNF $\alpha$ [42]. It additionally inhibits DC differentiation and maturation with preservation of an immature phenotype as evidenced by a decreased expression of MHC class II molecules, costimulatory molecules and IL12[43-45] (Figure 1B).

Inhibition of DC differentiation and maturation is particularly important in the context of autoimmunity and the abrogation of self tolerance. Antigen presentation to a T cell by a mature DC facilitates an immune response against that antigen while antigen presentation by an immature DC facilitates tolerance. Self-antigens are abundant in the normal state from physiologic cell death and turnover. However, presentation of these self-antigens is usually by immature DCs so that tolerance to self is maintained.

Given the importance of vitamin D for a functional immune system and the profound deficiency observed in autoimmune disease, as well as the correlation of deficiency with more active disease, an important issue is whether or not the immune components in autoimmune disease are capable of responding appropriately to vitamin D. Immune cells (B cells, T cells, monocytes, DCs) from multiple autoimmune diseases appear to respond to the immunomodulatory effects of vitamin D. Examples of vitamin D responsiveness by immunologic components in different autoimmune disease follow: B cells: Abnormalities of B cells from lupus patients may be partially reversed by vitamin D. Both spontaneous and stimulated immunoglobulin production from B cells from active lupus patients are significantly decreased by pre-incubating cells with 1,25 vitamin D[46]. Additionally, preincubation with vitamin D significantly decreases spontaneous production of anti-DNA antibodies by approximately 60%[46]. T cells: T cells from patients with MS respond to vitamin D. The proliferation of stimulated CD4 cells from MS patients and controls are

similarly inhibited after preincubation in increasing concentrations of vitamin D[27]. Moreover, Th17 polarized T cells from both controls and MS patients respond when incubated with vitamin D; both are downregulated with diminished production of IL-17 and gamma interferon[27]. Monocytes: Vitamin D inhibits the production of inflammatory cytokines (IL-1, TNF $\alpha$ ) by monocytes. Cytokine production by monocytes from both normal controls and from patients with autoimmune diabetes (type 1 or latent autoimmune diabetics) is significantly diminished by vitamin D[47]. TLR 4 stimulation by LPS or LTA (leipoteichoic acid) is similarly inhibited by exposure to vitamin D[47]. DCs: Lupus DCs are susceptible to the effects of vitamin D. LPS induced DC maturation is inhibited by preincubation with vitamin D resulting in suppressed expression of HLA class II and costimulatory molecules. The response of lupus cells to LPS stimulation is similarly suppressed by vitamin D[48]. Furthermore, vitamin D affects the expression of the interferon (IFN) signature in SLE. Interferon is produced by plasmacytoid DCs; the IFN signature refers to the overexpression of IFN  $\alpha$  inducible genes in peripheral blood mononuclear cells (PBMCs) of lupus patients[49]. The signature occurs in approximately 50% of patients and correlates with disease activity[50-52]. We have observed that interferon inducible genes are overexpressed in lupus patients with low serum vitamin D compared to normal serum vitamin D (Figure 2A). Expression of these interferon inducible genes may be diminished in lupus patients after receiving vitamin D supplementation (Figure 2B). In fact, we have observed that an IFN signature response, the decrease in expression of IFN inducible genes is 2.1 times more likely to occur in vitamin D supplemented lupus patients (unpublished data Ben-Zvi, I). There is currently a double-blind

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 placebo controlled NIH sponsored trial (ClinicalTrials.gov identifier: NCT00710021)  
 assessing the potential ability of vitamin D to suppress the interferon signature in patients with SLE.

Conclusions Vitamin D has important functions beyond those of calcium and bone homeostasis which include modulation of the innate and adaptive immune responses. Vitamin D deficiency is prevalent in autoimmune disease. Cells of the immune system are capable of synthesizing and responding to vitamin D. Immune cells in autoimmune diseases are responsive to the ameliorative effects of vitamin D suggesting that the beneficial effects of supplementing vitamin D deficient individuals with autoimmune disease may extend beyond effects on bone and calcium homeostasis.

Immunomodulatory effect of vitamin K2: Implications for bone health

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Abstract Objective—In women with postmenopausal osteoporosis, Vitamin K2 appears to decrease the incidence of hip, vertebral and non-vertebral fractures. Women with post-

menopausal osteoporosis have more circulating activated T cells compared to healthy post-menopausal and pre-menopausal women, but the effects of Vitamin K2 on T-cells has not been studied. In this study, we have looked at T-cell suppression by Vitamin K2.

**Materials and methods**—Peripheral blood mononuclear cells (PBMCs) from three healthy donors were used. The PBMCs were stimulated with the mitogens phytohemagglutinin and concanavalin A, and T-cell proliferation was analyzed using flow cytometry based on carboxyfluorescein succinimidyl ester (CFSE) dye dilution.

**Results**—Vitamin K2 (60 and 100  $\mu$ M) inhibited T-cell proliferation. Vitamin K1 at the same concentrations did not inhibit T-cell proliferation.

**Conclusion**—Vitamin K2 has immunomodulatory activities.

**Keywords** Vitamin K2; Immunomodulation; T-cells; Osteoporosis

### Introduction

Vitamin K is a fat-soluble vitamin. Two vitamin K species are known: vitamin K1 (phylloquinone), vitamin K2 (menaquinones). Vitamin K1 activates blood clotting factors. Vitamin K2, acts on extra-hepatic tissues (bone, brain, vasculature, testis, pancreas, kidneys and lungs) to activate K2 dependent proteins such as osteocalcin and matrix gla protein.

The most common forms of vitamin K2 in the human diet are MK-4 and MK-7, short- and longchained molecules, respectively (Beulens et al., 2013; Booth, 1997, 2012; Myneni and Mezey, 2016; Shearer and Newman, 2008). The effect of vitamin K2, mainly MK-4, on the

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**Author contributions** VM designed the study, analyzed the data and wrote the manuscript. EM reviewed the data and edited the manuscript.

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bone health of women with post-menopausal osteoporosis has been studied by a number of clinical investigators (Inoue et al., 2009; Iwamoto et al., 2003; Knapen et al., 2007; Tanaka and Oshima, 2007; Ushiroyama et al., 2002; Yonemura et al., 2004; Yonemura et al., 2000). A meta-analysis of studies in which MK-4 was used at a dose of 45 mg/day revealed that vitamin K2 reduced hip, vertebral and non-vertebral fractures (Cockayne et al., 2006).

Women with post-menopausal osteoporosis have more circulating activated T cells compared to healthy post-menopausal and pre-menopausal women (Adeel et al., 2013; D'Amelio et al., 2008). T-cells are known to play a critical role in promoting bone loss in postmenopausal osteoporosis as well as bone cancers (Zhang et al., 2011), rheumatoid arthritis (Kong et al., 1999), and periodontitis (Teng et al., 2000). Given the protective effect of vitamin K2 on bone health and the role of activated T-cells in osteoporosis, we asked if vitamin K2 could suppress T-cell proliferation. This could contribute to the activities described above. We report that vitamin K2 indeed can suppress T-cell proliferation. This activity is specific to vitamin K2, and not shown by vitamin K1.

**Materials and Methods** Reagents All reagents used in this study were obtained from Sigma-Aldrich (St Louis, MO) unless mentioned. CFSE, phytohemagglutinin and Concanavalin A

were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CD3 APC antibody and 7-AAD were purchased from eBioscience.

PBMC staining and culture Human PBMCs from healthy adult volunteers were obtained from the NIDCR core facility. Healthy volunteer blood was collected in accordance with the Declaration of Helsinki received from the NIH blood Bank. PBMCs were cultured in RPMI-1640 media with 10% Heat inactivated FBS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1× nonessential amino acids and 1% penicillin-streptomycin. PBMCs were stained with 2.5  $\mu$ M CFSE for 15 min at room temperature in the dark at a cell density of  $1 \times 10^6$  PBMC/ml. The reaction was stopped by adding an equal amount of cold RPMI-1640 media with 10% Heat inactivated FBS and incubated on ice for 5 min and centrifuged at  $400 \times g$  for 5 min. The cells are washed twice with cold media and resuspended at a density of  $1 \times 10^6$  PBMCs/ml. The CFSE labelled PBMCs were used for the T-cell proliferation assay. T-cell proliferation assay The CFSE-labeled PBMCs were cultured in triplicate at a density of  $5 \times 10^4$  cells/ml in a round bottom 96-well plate. T-cell proliferation was induced with phytohemagglutinin (PHA-5  $\mu$ g/ml) or concanavalin A (ConA-5  $\mu$ g/ml) for 96 hr with or without vitamin K2 (MK-4) or vitamin K1 in a total volume of 200  $\mu$ l. Cells were cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub>. T-cell proliferation was determined by CFSE dye dilution of CD3 positive cells using AccuriC6 flow cytometer. The gating strategy is shown in Supplemental Fig 1. The Flow cytometry data were analyzed using Flow Jo software.

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Statistical analysis All values are expressed as standard error of the mean (SEM) of three donors. Statistical significance was assessed by unpaired ANOVA followed by Tukey's post hoc testing using Prism 7. P values are as follows: \*\*\* $p > 0.001$ , \*\*\*\* $p > 0.0001$ .

#### Results and Discussion

In an initial series of experiments, we determined optimal concentrations of the mitogens PHA and ConA to promote T-cell activation using three donors to balance individual immune response variability (Supplemental Fig.2). A concentration of 5  $\mu$ g/ml was chosen for both PHA and ConA, and was used for the rest of the study. We initially tested the effect of vitamin K2 (MK-4) on T-cell proliferation at concentrations ranging from 0  $\mu$ M to 10  $\mu$ M, concentrations most commonly reported in literature to affect the function of cells such as osteoblasts (Ichikawa et al., 2007), osteoclasts (Koshihara et al., 2003), hematopoietic stem cells (Miyazawa and Aizawa, 2004), and erythroid and myeloid cells (Sada et al., 2010). Vitamin K2 at these concentrations did not inhibit T-cell proliferation (Supplemental Fig.3). Vitamin K2 concentrations greater than 10  $\mu$ M were shown to induce testosterone production (Ito et al., 2011). For the next set of experiments we used vitamin K2 concentrations as high as 100  $\mu$ M. Vitamin K2 significantly inhibited T-cell proliferation at 60 and 100  $\mu$ M in both PHA (Fig.1. a, b) and ConA (Fig.1. c, d) stimulated PBMCs. 30  $\mu$ M of K2 significantly inhibited ConA-stimulated, but not PHA-stimulated PBMCs (Fig.1. b, d). We next asked whether vitamin K1 also inhibits T-cell proliferation. As shown in Fig.2. a, b, c, d, vitamin K1 did not inhibit T-cell proliferation at any of the concentrations used. These results suggest that inhibition of T-cell proliferation is specific to Vitamin K2. It has been reported that vitamin K2, but not vitamin K1, promotes osteoblasts differentiation from mesenchymal stem cells, preventing osteoclast formation. This is

similar to the effect on T-cell proliferation that we observed. Our results also suggest that immunomodulation might contribute to the overall anabolic effect of vitamin K2 on bone. In clinical trials with vitamin K2, investigators used a dose of 45 mg/day. This is the minimal effective dose to improve bone health, and there were no side effects reported when it was given continuously for three years (Inoue et al., 2009; Iwamoto, 2014). In principal, this dose of K2 taken orally could achieve a maximum blood concentration of 40 to 50  $\mu$ M, but this needs to be further investigated. This would have clinical impact in that by modulating the dosage of vitamin K2 T-cell function can be modulated without affecting its functions on bone cells and other cell types. In this study, we have not looked at T-cells subsets, however. Doing this will provide a better understanding of the specific cell(s) that contribute to the function of vitamin K2 in promoting immunomodulatory activities. Further studies will be needed to determine the mechanism of immune regulatory functions of vitamin K2, and to examine the function of other vitamin K2 isoforms on immune regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Probiotic strain *Bacillus subtilis* CU1 stimulates immune system of elderly during common infectious disease period: a randomized, double-blind placebo-controlled study Marie Lefevre<sup>1\*</sup>, Silvia M. Racedo<sup>2</sup>, Gabrielle Ripert<sup>2</sup>, Béatrice Housez<sup>3</sup>, Murielle Cazaubiel<sup>3</sup>, Corinne Maudet<sup>3</sup>, Peter Jüsten<sup>1</sup>, Philippe Marteau<sup>4</sup> and Maria C. Urdaci<sup>2</sup>

Abstract Background: *Bacillus* probiotics health benefits have been until now quite poorly studied in the elderly population. This study aimed to assess the effects of *Bacillus subtilis* CU1 consumption on immune stimulation and resistance to common infectious disease (CID) episodes in healthy free-living seniors. Results: One hundred subjects aged 60–74 were included in this randomized, double-blind, placebo-controlled, parallel-arms study. Subjects consumed either the placebo or the probiotic ( $2.10^9$  *B. subtilis* CU1 spores daily) by short periodical courses of 10 days intermittently, alternating 18-day course of break. This scheme was repeated 4 times during the study. Symptoms of gastrointestinal and upper/lower respiratory tract infections were recorded daily by the subjects throughout the study (4 months). Blood, saliva and stool samples were collected in a predefined subset of the first forty-four subjects enrolled in the study. *B. subtilis* CU1 supplementation did not statistically significantly decrease the mean number of days of reported CID symptoms over the 4-month of study (probiotic group: 5.1 (7.0) d, placebo group: 6.6 (7.3) d,  $P=0.2015$ ). However, in the subset of forty-four randomized subjects providing biological samples, we showed that consumption of *B. subtilis* CU1 significantly increased fecal and

salivary secretory IgA concentrations compared to the placebo. A post-hoc analysis on this subset showed a decreased frequency of respiratory infections in the probiotic group compared to the placebo group. Conclusion: Taken together, our study provides evidence that *B. subtilis* CU1 supplementation during the winter period may be a safe effective way to stimulate immune responses in elderly subjects. Keywords: Clinical trial, Elderly, Common infectious disease, Probiotics, Immunostimulation

Background Viral respiratory and gastrointestinal infections are a predominant cause of morbidity and mortality in the elderly whose ageing immune system contributes significantly to poor outcomes [1]. Ageing is associated with a decline of innate and adaptive immune responses. For innate dysfunction, it has been described that the function of natural

killer cells, dendritic cells [2], macrophages [3] and neutrophils [4] decrease in the elderly. Moreover, age-dependent thymic involution leads to the reduction of circulating naive T cells and the increase frequency of regulatory, memory and effector T cells [5, 6]. A dramatic reduction in B cell repertoire associated with a decreased systemic antibody response to vaccination has been observed in the elderly population [7] showing that the B cell compartment is also affected by ageing [8, 9]. In addition, the production of secretory IgA (SIgA) at the mucosal surfaces decreases with age

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Lefevre et al. *Immunity & Ageing* (2015) 12:24 DOI 10.1186/s12979-015-0051-y and can lead to an increased risk of infection [10, 11]. SIgA, the predominant immunoglobulin class in human external secretions, is a key element in the maintenance of gut microbiota homeostasis and in the protection of gastrointestinal and respiratory tracts against pathogens [12]. Moreover, it has been also shown that the age-dependent modifications of the composition of the gut microbiota also contribute to the defective local and systemic immune defenses in the elderly population [13, 14]. The development of strategies aimed at counterbalancing the immune frailty in the elderly is a major challenge for 21st century medicine. Nutritional supplementation, including probiotics, in this population may help maintain immune function either by direct interaction with the host immune system or indirectly by re-equilibrating the gut microbiota [15–17]. Probiotics have been defined as ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host’ [18]. *Lactobacillus* and *Bifidobacterium* are the most commonly used bacterial probiotics. Fermented milk or dairy products containing *Lactobacillus* have shown effects on duration or frequency of respiratory and gastrointestinal infections [19–21] and reduced the risk of the common cold in healthy elderly subjects [22]. Mañé et al. [23] showed significant trends in reducing infection

incidence and mortality due to pneumonia in institutionalized elderly subjects treated with two *Lactobacillus plantarum* strains. Some trials showed that *Lactobacillus* and *Bifidobacterium* probiotics could increase influenza vaccination immune responses in the elderly [24–27]. Endospore formers such as *Bacillus* species are interesting because their spores resist the acid barrier of the stomach and are stable for long periods in commercial food products [28]. Bacilli, considered as gut commensals, have been used as probiotics for prophylaxis of human gastrointestinal disorders, to prevent recurrent respiratory infections or as an adjunct to antibiotic use [29–34]. Probiotics have been suggested to protect against infectious diseases by several strain-dependant mechanisms [35, 36] including secretion of anti-pathogen substances, competitive exclusion of pathogens, maintenance of mucosal integrity and stimulation of systemic or mucosal immune responses [35–39]. *Bacillus* species have been shown to produce antimicrobial substances [40, 41], to enhance epithelial gut barrier functions [42, 43] and stimulate cytokine [43–45] and SIgA in humans [46]. In this study we evaluated the beneficial effect of *Bacillus subtilis* CU1 administration in an elderly population. This probiotic displays immunostimulating properties and antagonizes gastrointestinal pathogen infection by producing antimicrobial substances such as amicoumacins (Racedo SM & Urdaci MC, unpublished observations). This randomized, double-blind placebo-controlled study investigated the effect of probiotic strain *B. subtilis* CU1 intake on resistance to common infectious disease (CID), notably by measuring mean cumulative number of days with CID in healthy free-living seniors (individuals over 60 years old). As secondary endpoints, the study examined the effect of *B. subtilis* CU1 intake on the stimulation of the mucosal and systemic immune response by measuring intestinal and salivary SIgA levels and serum cytokine levels, respectively, in a subset of 44 subjects.

**Results Subject characteristics** One hundred thirty two (132) subjects were screened for study eligibility, and 100 were randomized to the probiotic group (N=50) or to the placebo group (N=50) (Fig. 1). All enrolled subjects completed the study without major deviation. The baseline characteristics for the population are presented in Table 1. The data from the initial clinical examination were normal for all volunteers. Mean age observed in the probiotic group (63.3 (2.8) years of age) and placebo group (63.0 (2.4) years of age) were consistent with inclusion criteria. The influenza vaccination rates in the subjects, seasonal influenza and influenza A, from the beginning of the influenza vaccination season (September) were respectively 16.0 % and 8.0 % in probiotic group and 14.0 % and 12.0 % in placebo group. A good mean compliance was observed (>99 % in both groups).

**Clinical outcomes on the whole study population** Considering the whole study population (N=100), the mean number of days with CID symptoms over the 4month study period was 5.1 (7.0) d in the probiotic group and 6.6 (7.3) d in the placebo group (P=0.2015) (Table 2). The percentage of subjects reporting at least one CID episode during the study was 58.0 % in the probiotic group (N=29/50) and 66.0 % in the placebo group (N=33/50) (P=0.4106).

The risk to report an

Fig. 1 Flow chart of subjects

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infectious episode in the probiotic group was 12 % lower than in the placebo group (Relative Risk=0.88 [1.20;0.65]). There was no statistically significant difference between the probiotic and the placebo groups in mean duration, intensity, and frequency of CID during the observation period (P=0.2361, P=0.7400, and P= 0.3290 respectively).

Clinical outcomes on the subset of 44 subjects (post-hoc analysis) Considering the subset of the 44 subjects, the mean number of days with CID symptoms was 4.5 (7.3) days in the probiotic group and 7.3 (8.2) days in the placebo group (P=0.1101) (Table 2). In this subset, the percentage of subjects reporting at least one infectious episode during the study was 50.0 % (N=11/22) in the probiotic group and 72.7 % (N=16/22) in the placebo group (P=0.1260). The risk to report an infectious episode was 31 % lower in probiotic group than in placebo group (Relative Risk=0.69 [1.12;0.42]). There was no difference in mean duration, intensity and frequency of CID during the observation period between the probiotic and placebo groups (P=0.2361, P=0.7400, and P=0.1117 respectively). In the same subset (N=44), the frequency of respiratory infections was significantly lower in the probiotic group compared to the placebo group (P=0.0323): a mean number of 0.6 (0.7) respiratory infections was observed in the probiotic group vs. 1.1 (0.9) in the placebo group. The mean number of days with respiratory CID

Table 1 Baseline subject characteristics of the whole population (N=100), by product group

Probiotic group	Placebo group	(N=50)	(N=50)	Mean	SD	Mean	SD	Age (years)
63.3	2.8	63.0	2.4	Body weight (kg)	60.5	22.5	57.4	21.2
25.5	5.0	24.9	4.0	Body mass index (kg/m <sup>2</sup> )	25.5	5.0	24.9	4.0
80.0	78.0	CID during previous winter	2.7	1.0	3.2	1.2	Vaccination	Seasonal influenza n
87	16.0	14.0	Influenza A n	46	8.0	12.0	Pneumococcus n	00
0.0	0.0	0.0	0.0	Mean values and standard deviations; numbers and percentages				

Table 2 Effect of probiotic (*B. subtilis* CU1) and placebo consumption on clinical outcomes of infectious diseases

Whole population (N=100)	Subset of population (N=44)	Probiotic group (N = 50)	Placebo group (N = 50)	P	Probiotic group (N = 22)	Placebo group (N = 22)	P
Mean	SD	Mean	SD	Mean	SD	Mean	SD
5.1	7.0	6.6	7.3	0.2015a	4.5	7.3	7.3
7.3	8.2	0.1101a	Mean duration of CID (d)	5.0	4.6	5.3	4.1
0.2361a	Mean intensity of CID	8.1	5.0	7.6	4.4	0.7400a	9.0
6.2	8.8	5.3	0.7400a	CID frequency	1.0	1.2	1.2
1.2	0.3290a	0.8	1.0	1.3	1.2	0.1117a	Subjects with at least one CID
0.4106b	0.1260b	n	29	33	11	16	%
58.0	66.0	50.0	72.7	Mean number of days with RI	4.4	6.9	6.2
7.2	0.1027a	3.7	6.9	6.6	7.9	0.0818a	Mean duration of RI (d)
5.9	5.0	5.6	4.2	0.9043a	6.8	6.3	6.1
4.3	0.9325a	Mean intensity of RI	9.3	5.3	7.8	4.6	0.1428a
11.1	6.3	9.3	5.6	0.3473a	RI frequency	0.7	0.9
1.1	1.2	0.1181a	0.6	0.7	1.1	0.9	0.0323a
0.0323a	Subjects with at least one RI	0.1609b	0.0701b	n	24	31	10
16	%	48.0	62.0	45.5	72.7	Data are presented for the whole population (N=100) and the subset of population with biology analysis (N=44). (Mean values and standard deviations; numbers and percentages) Statistical differences were evaluated using Wilcoxon-Mann-Whitney's test or Savage's test according to the asymmetry of data (a), or logistic regression model (b)	

Mean number of days with CID 5.1 (7.0) 6.6 (7.3) 0.2015a 4.5 (7.3) 7.3 (8.2) 0.1101a Mean duration of CID (d) 5.0 (4.6) 5.3 (4.1) 0.2361a 5.8 (5.6) 5.7 (4.1) 0.2361a Mean intensity of CID 8.1 (5.0) 7.6 (4.4) 0.7400a 9.0 (6.2) 8.8 (5.3) 0.7400a CID frequency 1.0 (1.2) 1.2 (1.2) 0.3290a 0.8 (1.0) 1.3 (1.2) 0.1117a Subjects with at least one CID 0.4106b 0.1260b n 29 33 11 16 % 58.0 66.0 50.0 72.7 Mean number of days with RI 4.4 (6.9) 6.2 (7.2) 0.1027a 3.7 (6.9) 6.6 (7.9) 0.0818a Mean duration of RI (d) 5.9 (5.0) 5.6 (4.2) 0.9043a 6.8 (6.3) 6.1 (4.3) 0.9325a Mean intensity of RI 9.3 (5.3) 7.8 (4.6) 0.1428a 11.1 (6.3) 9.3 (5.6) 0.3473a RI frequency 0.7 (0.9) 1.1 (1.2) 0.1181a 0.6 (0.7) 1.1 (0.9) 0.0323a Subjects with at least one RI 0.1609b 0.0701b n 24 31 10 16 % 48.0 62.0 45.5 72.7 Data are presented for the whole population (N=100) and the subset of population with biology analysis (N=44). (Mean values and standard deviations; numbers and percentages) Statistical differences were evaluated using Wilcoxon-Mann-Whitney's test or Savage's test according to the asymmetry of data (a), or logistic regression model (b)

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symptoms was 3.7 (6.9) in the probiotic group and 6.6 (7.9) in the placebo group (P=0.0818).

Immunological parameters (subset of 44 subjects) Mucosal response Fecal and salivary SIgA response. SIgA is widely used as marker of mucosal immunity in clinical studies [47]. Remarkably, we observed a significantly higher concentration of SIgA in stools in the probiotic group compared to the placebo group after 10 d of product consumption (probiotic group: 2062.6 (1161.8) µg/ml; placebo group: 1249.5 (863.8) µg/ml; P=0.0038) (Fig. 2). The increased SIgA levels were still observed at the end of consumption and 18 d after the end of *B. subtilis* CU1 consumption (probiotic group: 2424.4 (1252.3) µg/ml;

placebo group: 1297.1 (953.7)  $\mu\text{g/ml}$ ;  $P=0.0032$ ). Furthermore, fecal SIgA concentrations significantly increased between pre- and post-supplementation with *B. subtilis* CU1 after 10 days of probiotic consumption ( $P=0.0012$ ). Elevated levels persisted 18 d after the last probiotic consumption ( $P=0.0008$ ). SIgA concentrations in stools were not statistically affected by placebo consumption. In addition, a significantly higher concentrations of salivary SIgA were observed in the probiotic group compared to the placebo group at the end of consumption and 18 d after the end of *B. subtilis* CU1 consumption (probiotic group: 940.4 (446.0)  $\mu\text{g/mL}$ ; placebo group: 650.1 (343.5)  $\mu\text{g/mL}$ ;  $P=0.0219$ ) (Fig. 3).

Systemic response Blood IFN response (Fig. 4). No statistically significant difference in IFN-gamma concentrations were observed between groups, after 10 d of probiotic consumption (V1+10 d) (probiotic group: 9.7 (8.1)  $\text{pg/mL}$ ; placebo group: 31.8 (92.4)  $\text{pg/mL}$ ;  $P=0.0981$ ). However, IFN-gamma concentrations significantly increased in the probiotic group from first pre- to post- supplementation, after 10 d of probiotic consumption (probiotic group at V1: 6.9 (5.0)  $\text{pg/mL}$ ;  $P=0.0090$ ), while no significant change was observed in the placebo group. No statistically significant differences in the plasma concentrations of cytokines (IL-1beta, IL-4, IL-6, IL-8, IL-10, IL-12p70, IgA, and TNF-alpha) were measured between the probiotic and the placebo groups from pre- to post- supplementation.

Numeration of *B. subtilis* in the stools (subset of 44 subjects) An increase in *Bacillus* spore concentrations were observed in stools of subjects from the probiotic group but not in the placebo group (Table 3). Molecular typing using the OPL12 primer showed the presence of the *B. subtilis* CU1 strain in stools as opposed to the placebo group and demonstrates the viability of the probiotic strain.

Stool cytotoxicity (subset of 44 subjects) No statistically significant difference was measured in the cytotoxicity levels in stools between the

Fig. 2 Concentrations of secretory IgA in stools. Fecal SIgA concentrations were assessed in subjects from the subset of population ( $N=44$ ), at baseline (V1), after 10 days of consumption of study products (V1+10 d) and at the end of the study (V3). Values are means, with standard error of means represented by vertical bars. Fecal SIgA concentrations were significantly higher in the probiotic group compared to the placebo group (\*\* $P < 0.01$ ), and significantly increased in the probiotic group during the study (††  $P < 0.01$ , †††  $P < 0.001$ )

Fig. 3 Concentrations of secretory IgA in saliva. Salivary SIgA concentrations were assessed in subjects from the subset of population ( $N=44$ ), at the end of the study (V3). Values are means, with standard error of means represented by vertical bars. Salivary SIgA concentration was significantly higher in the probiotic group compared to the placebo group (\* $P < 0.05$ )

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probiotic group (94.25 % (34.46) of viability) and placebo group (93.98 % (31.06) of viability), after 10 d of product consumption ( $P=0.6328$ ). These data confirm the absence of stool cytotoxicity induced by *B. subtilis* CU1.

Safety (whole study population) Investigators reported 108 adverse events in the probiotic group and 85 in the placebo group ( $P=0.8369$ ). There were no abnormal values of biological parameters at the end of the study, and no clinically significant variation was observed during the study, on renal and hepatic functions.

Discussion The current controlled study was designed to evaluate the effect of probiotic strain *B. subtilis* CU1 consumption (2.109spores/d) on immune system stimulation and

resistance to respiratory and gastrointestinal CID in healthy free-living seniors with known past histories of CID during the winter period. The demographic characteristic of the volunteers, the duration of study and the compliance to product consumption were similar for probiotic and placebo groups. The probiotic product was safe and well tolerated. An increase in *B. subtilis* CU1 concentration was observed in stools after intake of the probiotic product, which suggests survival of the strain in the gastrointestinal tract and is consistent with high compliance of study assessed by product consumption. The probiotic did not significantly affect CID in the whole population (N=100). In the subset of 44 subjects, and as planned in the protocol, biological explorations were performed. These analyses showed that *B. subtilis* CU1 significantly increased the levels of SIgA in stools (P=0.0032) and saliva (P=0.0219) in comparison to placebo and induced significantly higher levels of serum IFN-gamma (P=0.0090). Furthermore, a post-hoc analysis in this subset of subjects showed a statistically lower frequency of respiratory infections in the probiotic group compared to the placebo group (P=0.0323). We readily acknowledge several limitations to our study. Except for respiratory infections in the subset of 44 subjects, the clinical efficacy of oral administration of probiotic *B. subtilis* CU1 did not reach statistical significance. Consequently the study hypothesis was not reached, i.e., to observe a difference of 3 d in CID episodes between the two groups. However the statistical power of our study was lower than expected and *B. subtilis* supplementation tended to decrease the mean number of days with respiratory CID symptoms compared to the placebo group in the subset of 44 subjects (post-hoc analysis). In addition, *B. subtilis* CU1 supplementation significantly reduced the frequency of respiratory infections in this subset of population (P=0.0323). This subgroup was originally planned in the protocol only for biological analysis and the clinical efficacy observed in this subset, while not confirmed in the whole population, might be explained either by chance only or by the higher infection rates at the beginning of the clinical study (i.e., beginning of winter season, which is likely to correspond to the highest CID exposure period).

*B. subtilis* CU1 stimulates systemic immune response In the present study, we observed that supplementation with *B. subtilis* CU1 stimulated systemic immune response in seniors by significantly increasing serum IFN-gamma in the probiotic group following first *B. subtilis* supplementation. Concentrations of other measured

Fig. 4 Concentrations of IFN-gamma in blood. IFN-gamma concentrations were assessed in subjects from the subset of population (N=44), at baseline (V1), after 10 days of consumption of study products (V1+ 10 d) and at the end of the study (V3). Values are means, with standard error of means represented by vertical bars. IFN-gamma concentrations were significantly increased in the probiotic group between V1 and V1+10 d (††P <0.01)

Table 3 Bacterial counts of *Bacillus* spores in the stools of the subset population (N=44)  
 Whole population (N=100) Probiotic group (N=22) Placebo group (N = 22) Mean SD Mean SD  
 V1 2.5.103 3.0.103 2.5.103 5.4.103 V1+10 days 1.9.107 1.1.107 2.3.103 5.1.103 V3  
 7.5.103 1.5.104 3.0.103 4.4.103 Mean values and standard deviations

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serum cytokines and serum IgA were not significantly modified. These results are in accordance with previous mouse studies using *B. subtilis* CU1 (Racedo SM & Urdaci MC, unpublished observations). Huang et al. [48] also found that *Bacillus* strains could stimulate systemic and intestinal IFN-gamma production in mice. This ProTh1 cytokine

plays a role in the host defense against several infectious diseases, including viral infection and has a variety of immune functions such as stimulation of macrophages and natural killer cells [49, 50]. Different studies have emphasized the importance of IFN-gamma production for the protective effect of probiotics against influenza infection [51, 52]. Further investigations in *B. subtilis* CU1's capability in increasing serum IFN-gamma levels and the strengthening of the systemic anti-viral and anti-bacterial immune defenses in the elderly population would be interesting.

*B. subtilis* CU1 enhances intestinal and respiratory mucosal immune responses An important finding of the present study was that, compared to placebo, the oral intake of *B. subtilis* CU1 resulted in higher SIgA production in the healthy seniors. Ten days of probiotic intake were sufficient to increase stool SIgA levels 65 % in treated seniors compared to placebo. Moreover, an increased level of 87 % was maintained at least 18 days after the last probiotic administration. Previous controlled clinical trials have shown that the intake of probiotic bacteria (mainly *Lactobacillus* and *Bifidobacterium*) stimulates mucosal immune systems by enhancing fecal IgA [53, 54]. Kabeerdoss et al. [54] observed an increase of fecal IgA during probiotic intake with a subsequent decrease after cessation of administration of the probiotic. We assessed SIgA concentrations in saliva at the end of the 18-d follow up period. *B. subtilis* CU1 intake produced a 45 % increase in SIgA relative to placebo group. Since *B. subtilis* CU1 had been shown to increase IgA producing B cells in Peyer's patches in mice (Racedo SM & Urdaci MC, unpublished observations), one can postulate that *B. subtilis* CU1 consumption strengthens the generation of  $\alpha 4\beta 7$ +IgA+ B cells in the Peyer's patches of the small intestine of elderly subjects. The homing of these IgA producing B cells to the intestinal mucosa and the salivary glands [55] most probably accounts for the high SIgA levels measured in the feces and saliva of the probiotic group. In the elderly, only one previous study has shown that oral administration of heat-killed *Lactobacillus pentosus* b240 produced an increase in salivary IgA secretion [16]. Importantly, it has been shown, in mice, that 50 % increase production of intestinal SIgA to a bacterial toxin significantly increased the vaccine-induced protection directed against the toxin [56]. Additionally, it has also been shown that a 20 % increase in production of total SIgA in saliva is associated with a decrease in the incidence of colds and flu-like symptoms in humans [57, 58]. Therefore, increased SIgA levels of 87 % and 45 % in faeces and saliva respectively are most probably of physiological significance in ameliorating the health status of seniors receiving *B. subtilis* CU1. Taken together, these findings show the utility of oral administration of probiotic *B. subtilis* CU1 to increase mucosal immune responses. The increased SIgA levels in the intestine and saliva might contribute to strengthening the mucosal anti-viral and anti-bacterial immune defenses of the elderly population. It has to be noted that secretion of salivary SIgA has been shown to be impaired by stress such as academic stress or intensive physical exercise [59, 60]. Therefore interesting future work would be to investigate whether *B. subtilis* CU1 stimulation of mucosal immune system might be beneficial in the general population, notably in a population under stress. King et al. [61] recently published a meta-analysis and systematic review showing that *Lactobacillus* spp. and *Bifidobacterium* spp. strains brought by food products or supplements significantly lowered the number of days of acute respiratory infections in a healthy population of children and adults and shortened acute respiratory infectious periods. There are only few studies in the elderly and the present one is the first to indicate a trend toward a reduction of CID by a *B. subtilis* strain in this

population. Additional larger clinical trials have to be conducted to confirm these clinical outcomes.

**Conclusions** Despite the absence of significant results on CID in the whole population, the present study showed that consumption of *B. subtilis* CU1 significantly increased intestinal and salivary SIgA and serum IFN-gamma levels in a subset senior population. It suggests that daily *B. subtilis* CU1 supplementation during the winter months may be a safe effective way to stimulate systemic and mucosal immune responses of the elderly. However, no firm conclusion can be made about the effect of *B. subtilis* CU1 supplementation on CID.

Additional larger clinical trials have to be conducted to confirm these clinical outcomes.

**Methods** **Ethics, consent and permissions** The study was approved by the West IV Ethics Committee for Human Research and the French Health Products Safety Agency (AFSSAPS), France and was performed in accordance with the principles of the Declaration of Helsinki, of Good Clinical Practice (Directive ICH E-6, 24 November 2006), and current French regulations (Code de Santé Publique, Titre II du livre Premier). All participants Lefevre et al. *Immunity & Ageing* (2015) 12:24 Page 6 of 11

had given their written informed consent before inclusion in the study.

**Study subjects** One hundred (100) healthy free-living adults between 60 and 74 years of age, without any known congenital or immune defects, severe chronic disease or allergies and reporting at least two CID episodes during the previous winter period were recruited. Subjects were not included if any of the following criteria applied: chronic respiratory insufficiency, cardiac insufficiency, cancer (chemotherapy, radiotherapy), unstabilized chronic disease (severe renal or hepatic insufficiency, etc.), or any chronic severe affection likely to interfere with evaluation of the study parameters. For the inclusion, subjects were not allowed to take any drug potentially known to interfere with the evaluation of the study parameters, including corticoids and immunosuppressive drugs. Consumption of dietary supplements was forbidden in the last 2 months before inclusion, as well as regular consumption of probiotic products 1 month before the start of the study.

**Study design** The study was a monocentric, randomized, double-blind, placebo-controlled, parallel arms trial. Subjects were randomly allocated to the probiotic group or to the control group. The study lasted 16 weeks and consisted of four consumption periods of 10 d each, followed by 18 d without consumption of the study products (break period). At the selection visit, 1 or 2 weeks before initiation of the study, volunteers underwent a clinical examination and blood was taken from volunteers to assess safety parameters and measure of biological inclusion parameters. The investigators checked for inclusion criteria and recorded the subject's demographic characteristics. Eligible volunteers were randomly allocated by the investigator to the probiotic or the placebo group on Visit 1 (V1) of the study, according to the randomization list. Randomization was done without stratification using SAS® software version 9.1.3 Service Pack 4 (SAS Institute Inc., Cary, NC, USA). The randomization list was prepared before the beginning of the study by a person not related to the clinical phase, the data management or statistics. In addition, it was prepared and stored confidentially. The unblinding envelopes were concealed from the person responsible for randomization. During the study, four visits were planned: V1 (inclusion visit, beginning of the 1st product consumption period), V1+10 d (follow-up visit, 10 days after V1, end of the 1st product consumption period), V2 (follow-up visit, 2 months after V1) and V3 (end-of-study visit, 4 months after V1) (Fig. 5). The trial was conducted during the winter season 2010–2011, in the Nantes area, France.

**Study products** Study products were presented in the form of food supplements. The probiotic product consisted of *B. subtilis* CU1 mixed with excipients (maltodextrin DE14, dicalcic phosphate, magnesium stearate, colloidal silica). Each probiotic capsule contained 2.109 spores of *B. subtilis* CU1 (LESAFFRE, Marcq en Baroeul, France). The probiotic preparation contains 95 % of *B. subtilis* spores and 5 % of vegetative cells. Due to spore stability over time, the probiotic counts were the same at beginning and at the end of study. The placebo capsule contained only the excipient mix. Placebo products were indistinguishable from the probiotic product in appearance, smell and taste. During the four 10-d product consumption periods, the subjects were instructed to consume daily one capsule of the study product, in the morning (40 min before breakfast). Otherwise, subjects were asked not to modify their food habits and they were prohibited from taking any dietary supplement or food product containing probiotics during the study.

**Study endpoints** The primary endpoint was a between products comparison of the mean cumulative number of days with CID (upper and lower respiratory tract infections, gastrointestinal tract infections) during the 4 months of study in the whole population (N=100). In addition, some clinical endpoints were analysed in the whole population, and biological endpoints in the subset of the first

Fig. 5 Study design. R indicates randomization of the 100 subjects. Blood samples (B), fecal samples (F) and salivary samples (S) concerned a subset of 44 subjects

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44 subjects (half in the *B. subtilis* group, half in the placebo group). Clinical endpoints included the mean duration of CID, the intensity of CID, the frequency of CID, and the percentage of subjects with at least one CID (N=100). Biological endpoints included blood immunological marker concentrations (IL-1beta, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-alpha, IgA, IFN-gamma, at V1 and V1+ 10 d), salivary secretory IgA concentration (at V3), and fecal secretory IgA concentration (at V1, V1+10 d and V3) (N=44). At last, the presence of *B. subtilis* in stools and the cytotoxicity of stools were analysed in the subset population (N=44).

**Common infectious disease symptoms** During the 4 months of study, subjects were instructed to track any CID symptoms they had in a diary. The items detailed in this diary were: symptoms of gastrointestinal and upper/lower respiratory tract infections (cough, hoarseness, sore throat, itchy throat, rhinorrhea, sneezing, nasal obstruction, conjunctivitis, fatigue, headache, myalgia, nausea), body temperature, vomiting, and diarrhoea. The intensity of each symptom of gastrointestinal and upper/lower respiratory tract infections was rated on a 4-point scale (from 0: no symptom to 3: severe symptom) whereas fever (i.e., body temperature increased from at least one degree higher compared to basal temperature), vomiting and diarrhoea (i.e., more than 3 liquid stools per day) were rated on dichotomous scales (0=absence; 3=experience of the sign). All symptoms were reviewed by the investigator who determined if they complied with the diagnosis of a CID or not. When a CID was diagnosed, a symptomatic score was calculated on a daily basis, taking into account CID symptoms, fever, vomiting, and diarrhoea. This score rates from 0 to 45 arbitrary units. For analysis of the mean number of days with CID and the frequency of CID, all subjects were considered and "0" was applied to subjects without CID.

**Sample collection** Fasting blood samples and stool samples were collected at V1, V1+10 d and V3 among the first 44 randomized subjects (N=22 in probiotic group, N=22 in placebo group), which corresponded to the first twenty-two subjects enrolled in each group. The

randomized repartition of the subjects was ensured by the randomization list. Blood was centrifuged for 10 min at 1,000 x g at 4 °C to separate serum. An aliquot of the serum was used for IgA determination and a second aliquot was collected for cytokine analysis.

Additionally, saliva samples were collected at V3 in the same subset. Biological samples, serum, stools and saliva were stored frozen at -80 °C until analysis.

Serum, stools and saliva Immunoglobulin A (IgA) quantification Serum IgA concentration was measured by Elisa (enzyme-linked immunosorbent assay) according to the manufacturer's instructions (Hitachi 911, Roche, Basel, Switzerland). SIgA in stools and saliva were measured by Elisa (Immuchron AG, Heppenheim, Germany) according to the manufacturer's instructions.

Serum cytokine quantification Interleukins (IL-1beta, IL-6, IL-8, IL-10, IL-12p70), Tumor Necrosis Factor alpha (TNF-alpha) and Interferon gamma (IFN-gamma) were measured using Human Th1/Th2 11plex FlowCytomix immunoassays (eBioscience, Inc) on the BD Accuri™ C6 Flowcytometer (BD Bioscience) and ELISA (Ready-SET-Go® eBioscience, Inc.). Furthermore, human Interferon beta (IFN-beta) was analyzed by Elisa (R&D Systems, Inc.). All assays were performed according to the manufacturer's instructions.

Bacillus spores counts in stools and CU1 strain identification Stools samples (2 g) were placed in Stomacher® 400 classic bags, diluted with 18 ml of sterile physiological saline water and homogenized for 2 min. Ten ml of dilutions were heated (80 °C for 10 min) in order to kill bacterial vegetative cells. Samples were serially diluted 1:10 in sterile saline and plated on Mueller- Hinton Agar plates (Difco BD Laboratory, Franklin Lakes, USA) for 24 h at 37 °C for spores counts. In order to identify CU1 colonies in plates, a molecular typing method RAPD (Randomly Amplified Polymorphic DNA) was used. Different types of colonies from every fecal sample were analyzed in triplicate using the OPL12 primer that generates a specific profile for the CU1 strain [62].

Assessment of stool cytotoxicity Stool samples (1 g) were diluted in 5 ml of Dulbecco's modified Eagle's minimum essential medium (DMEM). After homogenization, samples were centrifuged at 10000 g for 10 min, then filtered through a 0.2µm membrane filter and diluted 1/20 (volume/volume (v/v)) in DMEM media. Stool cytotoxicity was evaluated by quantifying Vero cell detachment. Vero cells were grown in DMEM containing 25 mM glucose (Sigma-Aldrich), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (Gibco), 1 % (v/v) nonessential amino acids (Sigma), penicillin (100 UI/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml) (Sigma). For maintenance purposes, cells were passed every 3 d, using Accutase Solution (Sigma). Monolayers were prepared in 48-well tissue culture plates (Greiner Bio One, Germany) by seeding 5.10<sup>4</sup> cells per well. Experiments and cell maintenance were carried out at 37 °C in a 5 %

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CO<sub>2</sub>-95 % air atmosphere. Fully confluent cells (3–4 d in culture) were used throughout. Briefly, confluent cells were co-incubated with the different stool samples at 37 °C for 2 h and then cells were washed with phosphate-buffered saline (PBS), fixed for 1 min with 2 % (v/v) formaldehyde in PBS, washed again in PBS and stained with 500 µl of crystal violet solution (0.13 % crystal violet, 5 % ethanol, and 2 % formaldehyde in PBS w/v/v) for 20 min at room temperature. After being exhaustively washed with PBS to remove stain excess, samples were treated for 1 h with freshly prepared 50 % ethanol in PBS (v/v) at room temperature. Absorbance was measured at 650 nm in a Thermo Max Microplate spectrophotometer reader. Percentage of attached cells was calculated as: 100 x (A/Ac),

where A is the absorbance of treated cells and  $A_c$  is the absorbance of untreated control cells. As positive control a diluted supernatant of *Clostridium difficile* VPI 10463 was used. Safety Adverse events were collected during the study by investigators and reported in the case report forms of each subject. The investigators had also to evaluate imputability of any adverse event to the study products.

Statistical analysis Based on previous pilot clinical studies, sample size was calculated to detect an intergroup difference of 3 d in CID episodes (with Standard Deviation (SD): 5 d), using a twotailed t-test at the significance level of 0.05. Forty-eight subjects were required in each group to provide 80 % statistical power. To account for potential drop-outs, it was planned to include fifty subjects in each group. Data were analysed using SAS® software version 9.1.3 Service Pack 4 (SAS Institute Inc., Cary, NC, USA). Results are expressed as Mean (SD). Significance was set at  $P < 0.05$ . The mean number of days with CID and other clinical outcomes (mean duration, intensity, and frequency of CID) were compared between groups using the Wilcoxon-Mann-Whitney's test or Savage's test (according to the asymmetry of data). The percentage of subjects with at least one CID was compared between group using a logistic regression model. Immunological variables with a normal distribution (plasma, salivary and fecal IgA, IL-6, IL-8, IL-10, IL-12, and stool cytotoxicity) were compared using Student's t-test for between-group analysis and Student's paired t-test for within-group analysis. For criteria which do not respect normal distribution (IFN- $\gamma$ , TNF- $\alpha$ , IL-1 and IL-4), a Wilcoxon-Mann-Whitney's test was applied for between group analysis, and completed with Savage's test for IL-1 and IL-4 due to dissymmetry of these biological parameters. Within-group analysis was performed using Wilcoxon's paired test. The number of subjects with at least one side-effect in each group (probiotic and placebo groups) was compared using Chi-square test. Following this primary analysis, some post-hoc analyses were performed. The first analysed the clinical outcomes reported in the subset of 44 subjects (number of days with CID, mean duration of CID, intensity of CID, frequency of CID, and percentage of subjects with at least one CID). The second post-hoc analysis applied on the clinical outcomes in subjects who reported at least one symptom of upper/lower respiratory tract infections, whatever this symptom was or not associated to a symptom of gastrointestinal infection. This latter analysis was performed in the whole population of 100 subjects and in the subset of 44 subjects. The statistical models were identical to the ones applied for the primary analysis.

#### Current Directions in Stress and Human Immune Function

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Abstract Psychological stress has been linked empirically with dysregulation of facets of the human immune system, yet these effects are not the same in every situation or population. Recent research has made strides towards understanding risk factors for immune dysregulation as well as why these risks occur. This review discusses mechanisms and mediators underlying the stress-immune relation, the role of context in determining whether an immunologic responses to stress is adaptive versus maladaptive, and the stress-immune relation in populations including children exposed to early adversity, older

adults, and individuals with clinical diagnoses. The reviewed work holds great promise for further elucidating the circumstances under which psychological stress has immunological consequences, and provides new directions for work in this field.

### Introduction

Stress is a broad concept that comprises challenging or difficult circumstances (stressors) or the physiological or psychological response to such circumstances (stress responses). In humans, among other species, one of the systems that responds to challenging circumstances is the immune system. Broadly, the immune system comprises cells, proteins, organs, and tissues that work together to provide protection against bodily disease and damage (see Box for explanations of relevant immunological parameters). Several facets of the human immune system have been empirically associated with stress. During acute stress lasting a matter of minutes, certain kinds of cells are mobilized into the bloodstream, potentially preparing the body for injury or infection during “fight or flight” [1]. Acute stress also increases blood levels of pro-inflammatory cytokines [2]. Chronic stress lasting from days to years, like acute stress, is associated with higher levels of pro-inflammatory cytokines, but with potentially different health consequences [3]. Inflammation is a necessary short-term response for eliminating pathogens and initiating healing, but chronic, systemic inflammation represents dysregulation of the immune system and increases risk for chronic diseases, including atherosclerosis and frailty [4]. Another consequence of chronic stress is activation of latent viruses. Latent virus activation can reflect the loss of immunological control over the virus, and frequent activation can cause wear-and-tear on the immune system [5].

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Interestingly, these responses may not be the same for everyone. Those who have experienced early adversity, for example, may be more likely to exhibit exaggerated immune reactions to stress [6, 7]. Currently, the field is moving toward a greater understanding of who might be most at risk for chronic inflammation and other forms of immunological dysregulation, and why. This question is important not only for health, but also for longevity, as evidence suggests that the immunological effects of chronic stress can advance cellular aging and shorten telomere length [8].

Meta-analyses provide a look backward at this research and summarize what has been learned about the relationship between stress and human immunity since it was first studied in the 1960s [1, 2, 9]. This review describes recent, groundbreaking work on the stress-immune relation in humans, including the immunological consequences of stress in early and late life, mediators of the stress-immunity link, ecological perspectives, and how the relationship between stress and immunity is manifest in clinical populations (see Figure).

Early life stress Stress that occurs early in development (e.g., maltreatment, poverty, and other adverse experiences) has immunological consequences that can be observed both in the near and long term after the stressor occurs. Early life stress (ELS) in children associates with immunological dysregulation, including low basal levels of cytokines that control immune responses [10]. When immune cells were stimulated in vitro (e.g., with tetanus toxoid), those cells from children who experienced ELS produced more pro-inflammatory cytokines [10]. Whereas much of the extant research focuses on maltreatment or poverty, a recent study into the effects of a less-studied adversity, bullying, also suggests that chronic peer victimization predicts a steeper increase in CRP from childhood into young adulthood [11]. EBV antibody levels in a younger adult sample were also found to differ based on the type, timing, and frequency of exposure to ELS. Individuals exposed to sexual abuse more than 10 times, as well as those physically abused starting between ages 3 and 5, had elevated levels of antibodies against EBV as adults, a signal of viral reactivation [12]. In adults, a meta-analysis of ELS and inflammation found a positive association between maltreatment and several inflammatory markers, with the most robust association for circulating CRP [13]. Recent work has investigated mechanisms linking ELS to immune alterations over time (e.g., self-control, adiposity, smoking, and stress; 14, 15] as well as examining inflammatory dysregulation as a pathway through which ELS affects adult disease prevalence and outcomes [16]. Finally, empirically based interventions to target immunological consequences of ELS are a necessary next step; recent evidence suggests the plausibility of such interventions to improve inflammatory profiles for youth raised in low-income families [17].

Stress, immunity, and aging As people age, they are less able to mount appropriate immune responses to stressors. These could be physical stressors, such as injury, or psychological stressors such as caregiving. In addition, psychological stress affects organisms in a manner similar to the effects of chronological age, and chronological aging coupled with chronic stress accelerates immunological aging [18]. Research has suggested that older adults are unable to terminate

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cortisol production in response to stress. Cortisol is ordinarily anti-inflammatory and contains the immune response, but chronic elevations can lead to the immune system becoming “resistant,” an accumulation of stress hormones, and increased production of inflammatory cytokines that further compromise the immune response [18]. Older adults often have to provide long term care for an ailing spouse or partner. Caregiving has been implicated in significantly lower antibody and cell-mediated immune responses after vaccination [19, 20]. Caregivers also experience longer wound healing times, lower lymphocyte proliferation, increased proinflammatory cytokine levels, and more reactivation of latent viruses [21].

An important direction in aging research involves an examination of telomeres. Telomere length has been used as a measure of biological aging and is associated with psychological, physiological, and social factors. Chronic stress is linked to shortened telomere length along with increased disease in older adults [22]. Socioeconomic factors such as marital status and income have been linked with telomere length: those married for longer periods of time and who make more money are biologically younger than others in their cohort [22,

23]. However, studies thus far have found this link only in Caucasians and Hispanics, but not African Americans. This suggests that low socioeconomic status (SES) may accelerate aging in some populations [23]. Interestingly, health behaviors can moderate this effect by protecting individuals from accelerated aging during stress exposure [24]. It is unclear how this moderation occurs, and more work is needed.

Collectively, recent work points to new discoveries into how biological aging and stress interact to influence the immune response. This will lead to a better understanding of mechanisms of immunosenescence caused by stress and chronological aging that are presently unclear.

Biological and behavioral mediators of the relationship between stress and immunity How does stress get “under the skin” to influence immunity? Immune cells have receptors for neurotransmitters and hormones such as norepinephrine, epinephrine, and cortisol, which mobilize and traffic immune cells, ideally preparing the body to mount an immune response if needed [25]. Recent evidence shows that immunological cells (e.g., lymphocytes) change their responsiveness to signaling from these neurotransmitters and hormones during stress [26]. However, immunological responses are biologically and energetically costly, and over time, chronic stress produces negative systemic changes both in immune trafficking and in target tissues [6].

The linkages between stress and immunity may be mediated by specific health behaviors, psychosocial factors, or both. For instance, stress has been linked to being in troubled relationships, having negative or competitive social interactions, and feeling lonely, which have each in turn been linked to increases in pro-inflammatory responses to stress [27-29]. Other potential mediators, like getting good sleep, are increasingly being recognized as important pieces of the stress-immunity puzzle [30]. Even one night of total sleep deprivation was recently found to significantly increase neutrophil counts and decrease neutrophil function in healthy men [31].

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Taken together, these examples highlight a better understanding of the factors that mediate or moderate stress's influence on immunity. This direction may serve to one day develop targeted behavioral or pharmacotherapies to those at highest risk for poor health outcomes.

Ecological immunology Over the last several years, there has been greater attention paid to the relevance of ecological immunity to the relationship between stress and immunity.

Ecological immunity is based on the premise that mounting immune responses is energetically costly and that the (mal)adaptiveness of immune responses to stress is determined by cost:benefit ratios [32-34]. In early human history many stressors were life-threatening: being eaten by a predator, being excluded by one's peer group, or being faced with starvation, to name a few. Appropriately responding to some of these stressors (e.g., predation) required activating the energetically costly fight or flight response, including immunological changes that could protect against infection secondary to wounding.

However, energetic costs of the immune system during other kinds of stressors (e.g., social exclusion) that resulted in less availability of energetic resources (e.g., shared food) might have been counterproductive. Thus, downregulating immune responses might have been evolutionarily adaptive. Research in bumblebees finds that under conditions of starvation,

immune responses to an immune challenge accelerated time to death from starvation, suggesting that allocating energy to the immune system under those conditions was maladaptive [35]. Although energetic resources are abundant in the modern environment, physiological evidence of these ecological tradeoffs in the ancestral environment can still be found. For example, in contemporary humans, costly endeavors such as building and maintaining a large social network or persisting on unsolvable challenges can be associated with decreases in some immune parameters [36, 37]. Taken together, these and other findings [for reviews, see 33, 38] suggest that ecological conditions and resource availability may shape immune functioning in ways that remain relatively underexplored. Stress, immunity, and clinical health Psychological stress has been implicated in altered immune functioning in many diseases. Stress induces chronic immune activation and altered health outcomes that resemble those seen in chronic inflammatory diseases such as RA [39, 40]. Altered immune function can lead to exacerbated symptoms of both physical and psychological illnesses. In irritable bowel syndrome, sustained cortisol activity during stress is associated with an increase in gastrointestinal symptoms [41]. High levels of proinflammatory cytokines resulting from stress have recently been implicated in the etiology of schizophrenia and schizophreniarelated brain alterations [42]. Chronic stress has been shown to enhance risk for developing autoimmune disease [e.g., 43]. Individuals with autoimmune disease also appear to have difficulty down-regulating their immune responses after exposure to stressors. In MS, neuropeptides secreted under stress (e.g., corticotropin-releasing hormone) activate glial cells in the brain to release inflammatory molecules that result in brain inflammation and worsen MS pathology [44]. Similar immune activation and symptom exacerbation is evidenced in those with other autoimmune diseases [40]. Currently, possible mechanisms by which autoimmune diseases alter individual responses to stress are being explored. This

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knowledge may lead to interventions that decrease stress-induced immune responses and improve outcomes in autoimmune diseases.

Conclusions and future directions Research on the immunological effects of stress has burgeoned over the past decade following Segerstrom and Miller's meta-analysis [1]. This research has explored new avenues, including the areas reviewed here, that show particular promise for illuminating the conditions under which stress impacts the immune system. Research on stressors occurring early (i.e., childhood and adolescence) and late (i.e., aging) in the lifespan have suggested that individuals exposed to chronic stressors (e.g., abuse, caregiving) can exhibit immune dysregulation that may be persistent and severe. Stressor qualities (e.g., type, timing) as well as individual characteristics that make individuals more or less susceptible to these effects are targets for future work.

Examinations of mediators and mechanisms of the stressimmune relation can also determine how and for whom exposure to stress impacts the immune response. Ecological immunology suggests that downregulating the immune response may sometimes be adaptive, and future work building from this perspective will help to further elucidate contexts in which immunosuppression may occur but progress toward superordinate goals is facilitated. Finally, research into the effects of stress on inflammation in clinical populations has demonstrated that stress exposure can increase the likelihood of

developing disease, as well as exacerbating preexisting conditions. Further work in this area may help to treat or even prevent morbidity. Overall, this area of research is broad, rapidly developing, and holds promise for improving human health.

**27. What makes up an ideal protocol to restore a damaged gut?**

- a. Consume spore-based probiotics
- b. Consume prebiotics
- c. Improve the gut mucosal layer
- d. Repair the epithelial barrier
- e. All of the above

Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers

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Institutional review board statement: The study was reviewed and approved by the UNT Institutional Review Board for Human Subjects Research.

Informed consent statement: Subjects provided written and oral consent to participate using an IRB-approved informed consent form specific to the study in question.

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Data sharing statement: None.

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**Abstract AIM** To determine if 30-d of oral spore-based probiotic supplementation could reduce dietary endotoxemia.

**METHODS** Apparently healthy men and women (n = 75) were screened for post-prandial dietary endotoxemia. Subjects whose serum endotoxin concentration increased by at least 5-fold from pre-meal levels at 5-h post-prandial were considered “responders” and were randomized to receive either placebo (rice flour) or a commercial sporebased probiotic supplement [Bacillus indicus (HU36), Bacillus subtilis (HU58), Bacillus coagulans, and Bacillus licheniformis, and Bacillus clausii] for 30-d. The dietary endotoxemia test was repeated at the conclusion of the supplementation period. Dietary endotoxin (LAL) and triglycerides (enzymatic) were measured using

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Prospective Study

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an automated chemistry analyzer. Serum disease risk biomarkers were measured using bead-based multiplex assays (Luminex and Milliplex) as secondary, exploratory measures.

**RESULTS** Data were statistically analyzed using repeated measures ANOVA and a P < 0.05.

We found that spore-based probiotic supplementation was associated with a 42% reduction in endotoxin ( $12.9 \pm 3.5$  vs  $6.1 \pm 2.6$ , P = 0.011) and 24% reduction in triglyceride ( $212 \pm 28$  vs

$138 \pm 12$ , P = 0.004) in the post-prandial period Placebo subjects presented with a 36% increase in endotoxin ( $10.3 \pm 3.4$  vs  $15.4 \pm 4.1$ , P = 0.011) and 5% decrease in triglycerides ( $191 \pm 24$  vs  $186 \pm 28$ , P = 0.004) over the same post-prandial period. We also found that sporebased probiotic supplementation was associated with significant post-prandial reductions in IL-12p70 ( $24.3 \pm 2.2$  vs  $21.5 \pm 1.7$ , P = 0.017) and IL-1 $\beta$  ( $1.9 \pm 0.2$  vs  $1.6 \pm 0.1$ , P = 0.020). Compared to placebo post supplementation, probiotic subject had less ghrelin ( $6.8 \pm 0.4$  vs  $8.3 \pm 1.1$ , P = 0.017) compared to placebo subjects.

**CONCLUSION** The key findings of the present study is that oral sporebased probiotic supplementation reduced symptoms indicative of “leaky gut syndrome”.

**Key words:** Metabolic endotoxemia; Chronic disease; Leaky gut syndrome; Probiotics; Multiplex; Cardiovascular disease; Inflammatory cytokines; High-fat meal challenge

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**Core tip:** Dietary or metabolic endotoxemia is a condition that affects approximately 1/3 of individuals living in Western society. It is characterized by increased serum endotoxin concentration during the first five hours of the post-prandial period following consumption of a meal with a high-fat, high-calorie content. The key findings of the present study, were that 30-d of oral spore-based probiotic supplementation reduced the incidence of dietary endotoxemia, which may be indicative of reduced gut permeability.

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INTRODUCTION Incidence of gastrointestinal (GI) distress and permeability has increased in prominence in modern society due in large part to the excessive consumption of highly processed, calorie dense, commercially available foods[1]. These same dietary choices coupled with low physical activity are believed to be the primary causes underlying the current obesity epidemic[2]. Recent efforts have focused on the use of over-the-counter probiotics (typically *Lactobacillus* and *Bifidobacterium*) to address symptoms associated with GI abnormalities[3-5]. The lay literature has generally identified a goal of improved “GI health”, but unfortunately this is so broadly defined that it is nearly impossible to identify a single research focus[6]. Further complicating matters is that probiotic supplementation does not yield consistent results[7,8]. We have speculated that if an individual doesn’t have a pre-existing GI abnormality then they would not be a “responder” to probiotic supplementation. Complicating oral probiotic supplementation efforts is the fact that few traditional probiotic supplements (i.e., *Lactobacillus* and *Bifidobacterium*) deliver fully viable bacteria to the small intestine[9,10]. Recently it has been speculated gram positive, spore-forming probiotic strains may be a good alternative because the endospores that encapsulate the strains are highly resistant to stomach acid, potentially resulting in the delivery of more viable probiotics to the small intestine[11,12]. Thus, it appears that two major limitations of the existing probiotic literature lie with an inability to identify “responder” subjects prior to enrollment and issues associated with viable probiotic delivery to the small intestine. Dietary or metabolic endotoxemia occurs when one’s dietary consumption causes disruption in either GI permeability, the microbiota profile, or both[1,2,4,13-15]. Dietary endotoxemia transiently increases systemic inflammation, which chronically may increase one’s risk of a variety of diseases[2]. Our laboratory and others have demonstrated that consumption of a single, highfat, high-calorie meal was associated with an increase in serum endotoxin, triglycerides, metabolic biomarkers, inflammatory cytokines, endothelial microparticles, and monocyte adhesion molecules[16-22]. The post-prandial time course varies for each biomarker, but generally the transient changes occur during the first five hours of the post-prandial period. Given the direct link between nutrition, microbiota, GI permeability, and disease risk, our laboratory and others have speculated that these changes represent an appropriate treatment target for a probiotic intervention[23,24]. To address known issues with sufficient probiotic delivery, we utilized a “spore-based” probiotic in the present study. According to the literature the biggest advantages of a “spore-based” probiotic is that it is composed of endospores which are highly resistant to acidic pH, are stable at room temperature, and deliver a much greater quantity of high viability bacteria to the small intestine than traditional probiotic supplements[11,12]. To our knowledge, the present study is the first attempt to clinically leverage the benefits of spore-based probiotics to improve health outcomes. The primary purpose of the present study was to determine if 30-d of spore-based probiotic supplementation reduced post-prandial endotoxemia and triglycerides.

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The study enrollment was unique in that we developed an additional level of screening to only enroll subjects who had dietary endotoxemia (i.e., responders). Our secondary purpose was to determine if other metabolic biomarkers and cytokines, known to change

after consuming a high-fat meal, would also be modified by 30-d of spore-based probiotic supplementation.

**MATERIALS AND METHODS** Determination of appropriate sample size All the procedures described in the present study were reviewed and approved by the University of North Texas Institutional Review Board (IRB) for Human Subject's Research. Subjects provided their written and verbal consent to participate before being enrolled in the study. The present study was completed following a completion of a preliminary proof of concept study in the laboratory (data not shown). From this data, we identified that only 2 of 6 subjects ("responders") had a measurable dietary endotoxemia response (i.e., at least a 5-fold increase from pre-meal values at 5-h postprandial). "Responder" subjects experienced a 30% reduction in serum endotoxin (effect size = 0.40) at 5-h post-prandial following a 30-d probiotic intervention (same probiotic used in the present study). Based on these criteria, we identified that we needed to enroll a minimum of  $n = 10$  "responders" in placebo and spore-based probiotic groups ( $n = 20$  total) in order to achieve at least 80% statistical power. Eighty subjects were screened for a dietary endotoxin response, and 25 "responders" were enrolled (Table 1) and matriculated through the study treatments (Figure 1).

**Additional subject screening** Prior to testing for the post-prandial endotoxemia response, subjects also completed a series of other tests to exclude for other pre-existing conditions. Screening included measurement of body composition (whole body DEXA scan; GE Lunar Prodigy, United States), medical history assessment, and resting metabolic rate (RMR; MGC Diagnostics Ultima; St. Paul, MN, United States). Subjects who were currently taking or had taken in the previous 6-mo medications for the treatment of metabolic disease, antibiotics, probiotic supplements, anti-inflammatory medications, and/or daily consumed at least 3 serving of yogurt were excluded from further participation. Within the medical history, we also excluded subjects who were currently being treated for metabolic disease (i.e., diabetes mellitus), currently being treated for cardiovascular disease, and/or were obese (by BMI and/or percent body fat from DEXA). Individuals who met the initial screening criteria were scheduled to consume the experimental meal challenge on a separate day. The experimental meal challenge was used to identify subjects with a dietary endotoxin response that we considered "responders". Individuals classified as "responders" were enrolled in the supplementation phase of the study.

**Identification of "responders"** Experimental meal challenge: Subjects reported to the laboratory between 0600 and 1000 following an overnight fast (> 8-h) and abstention from exercise (> 24-h). Following collection of a pre-meal blood sample, subjects were provided a high-fat meal (85% of the daily fat RDA and 65% of the daily calorie needs based on RMR). Thin crust cheese pizza from a local vendor was used as the high-fat meal source (Table 2). Blood samples were measured for endotoxin concentration after the meal and only those subjects

Expressed interest (  $n = 137$ )

Scheduled for laboratory screening (  $n = 123$ )

Enrolled (  $n = 80$ )

Randomized (  $n = 32$ )

Placebo (  $n = 13$ )

Probiotic (  $n = 15$ )

Excluded, pre-screening ( n = 47)

Excluded, lab screening ( n = 43)

No dietary endotoxemia ( n = 48)

Non compliant ( n = 4)

Overall compliance 95%

Figure 1 Represents the consort diagram for the study that indicates the number of participants that matriculated through the study. Subjects were carefully screened for exclusion/inclusion criteria and if qualified were enrolled in the study. Consistent with our preliminary data 2 out of every 6 subjects presented a dietary/metabolic endotoxin response following consumption of the high-fat meal. A total of 26 individuals were identified to have the “responder” phenotype and were randomized to participate in either the probiotic or placebo condition.

Table 1 Subject characteristics

Characteristic Placebo ( n = 13)

Probiotic ( n = 15)	Age (yr)	21.8 ± 0.7	21.2 ± 0.5	Height (cm)	167.9 ± 3.2	170.8 ± 2.7	Body mass (kg)	74.2 ± 6.6	71.2 ± 3.1	Body mass index (kg/m <sup>2</sup> )	25.9 ± 1.5	24.3 ± 0.9	Body fat (%)	27.8 ± 4.1	25.2 ± 3.0	Fat mass (kg)	21.0 ± 4.3	17.3 ± 2.4	Lean mass (kg)	50.1 ± 3.8	50.0 ± 3.7	Bone mineral mass (kg)	2.9 ± 0.2	2.9 ± 0.1	Resting energy expenditure (kcal/d)	2243 ± 304	2071 ± 108
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Values represent group mean ± SEM. No significant differences existed between groups with respect to subject characteristics.

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whose endotoxin level increased by > 5-fold at 5-h post-prandial were classified as “responders” and enrolled in the supplementation phase of the study. This same experimental meal challenge was completed at the end of the supplementation period to assess the effectiveness of spore-based probiotic supplementation at modifying the serum endotoxin response.

Supplementation conditions: “Responder” subjects were randomized to either a placebo (rice flour) or spore-based probiotic (Megasporebiotic; Physicians Exclusive, LLC; Glenview, IL, United States) condition. The spore-based probiotic included 4 billion spores from gram-positive, spore-forming strains [Bacillus indicus (HU36), Bacillus subtilis (HU58), Bacillus coagulans, and Bacillus licheniformis, Bacillus clausii]. Subjects were instructed to consume 2 capsules each day for a total of 30-d. Subjects were asked to promptly report any missed doses. Based on subject reporting, efficacy of intake was > 95% for the study period. All group assignments were completed using double-blind procedures. Subjects were instructed to maintain their habitual dietary and lifestyle habits during the study.

Blood sample collection: Venous blood samples were collected prior to the high-fat meal (PRE), 3-h, and 5-h post meal from a peripheral arm vein into an evacuated serum tube. Serum tubes were held at room temperature for 30-min to allow for clotting. Serum was separated by centrifugation and frozen at -80 °C until additional analysis.

Dietary endotoxin measurement Serum was analyzed for endotoxin concentration using a commercially available kinetic limulus amoebocyte lysate (LAL) assay (Lonza; Allendale, NJ, United States). Briefly, serum samples were diluted 1:100 in endotoxin-free water and

heated at 70 °C for 15-min to remove contaminating proteases. Treated samples were then analyzed in triplicate using an automated chemistry analyzer (Chem Well T; Palm City, FL, United States) to determine endotoxin concentration against an *E. coli* endotoxin standard.

Serum triglyceride measurement Serum was analyzed in triplicate for triglyceride concentration using an endpoint enzymatic assay (Pointe Scientific; Canton, MI, United States) on an automated chemistry analyzer (ChemWell T).

Exploratory disease risk biomarkers: Previously frozen serum samples were analyzed as previously described[25-27]. Briefly, ghrelin, insulin, leptin, MCP-1, GMCSF, interleukin (IL)-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL12(p70), IL-13, and tumor necrosis factor alpha (TNF- $\alpha$ ) were measured in duplicate using a commercially available bead-based multiplex assay (Milliplex; MilliporeSigma; St. Louis, MO, United States) and an automated analyzer (Luminex MagPix; Austin, TX, United States). Raw data files were used to calculate unknowns from standards using Milliplex Analyst software (MilliporeSigma).

Statistical analysis Prior to formal statistical testing data were assessed for normality.

Non-normal data was log-transformed to stabilize this assumption prior to formal testing.

Data were analyzed using a condition (placebo or probiotic)  $\times$  experiment time (baseline and 30-d post)  $\times$  meal time (pre, 3, and 5-h post) analysis of variance (ANOVA) with repeated measurements on the 2nd and 3rd factors. P-values were adjusted using the Huynh-Feldt method to account for the repeated measures design. Significance was set at  $P < 0.05$ . Location of significant effects was determined using separate t-tests with a Bonferroni correction for multiple comparisons. In order to visualize the responses collectively, we log transformed all the responses to normalize the various biomarkers to a similar scale. We then created three radar plots (one for each sampling time point). Each plot contained the log transformed variable response at baseline and 30-d post and a third line for the fold-change from pre-meal response). Heat maps were generated for variables that showed similarity to endotoxin responses using a three-color approach: Red (large increase from pre-meal), yellow (intermediate response), and green (large decrease from pre-meal) (Figure 2). We have used a similar approach to data visualization in past manuscripts and this is an effective and accepted method[19,28].

**RESULTS** Endotoxin and triglycerides We found significant three-way interaction effects for both serum endotoxin ( $P = 0.011$ ; Figure 3A) and triglycerides ( $P = 0.004$ ; Figure 3B). In each instance, there was no difference between the post-prandial response between the two treatment groups (i.e.,

Table 2 Meal composition

Component Placebo ( n = 13)

Probiotic ( n = 15)	Total calories (kcal)	1630.4 $\pm$ 134.4	1644.7 $\pm$ 94.5	Total caloric needs
	(% of RMR)	72%	79%	Servings (#)
		6.3 $\pm$ 0.5	6.4 $\pm$ 0.4	Fat (g)
		88.8 $\pm$ 7.3	89.6 $\pm$ 5.1	Fat (kcal)
		799.3 $\pm$ 6.6	806.4 $\pm$ 46.3	Saturated fat (g)
		31.7 $\pm$ 2.6	32.0 $\pm$ 1.8	Trans fat (g)
		0	0	Protein (g)
		69.8 $\pm$ 5.8	70.4 $\pm$ 4.0	Carbohydrate (g)
		145.9 $\pm$ 12.0	147.2 $\pm$ 8.5	Carbohydrate (kcal)
		583.6 $\pm$ 48.1	588.8 $\pm$ 33.8	Cholesterol (mg)
		152.3 $\pm$ 12.5	153.6 $\pm$ 8.8	Sodium (mg)
		2911.9 $\pm$ 240.0	2937.4 $\pm$ 168.8	

Values represent group mean  $\pm$  SEM. No significant differences existed between groups with respect to meal composition.

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placebo vs spore-based probiotic) at baseline; however, the significant differences were apparent at postsupplementation. Specifically, spore-based probiotic supplementation was associated with a 42% reduction in serum endotoxin at 5-h post-prandial compared to a 36% increase in placebo at the same time point. Sporebased probiotic supplementation was associated with a 24% reduction in serum triglycerides at 3-h postprandial compared to a 5% reduction in placebo at the same time point.

**Exploratory biomarkers** We found significant trial  $\times$  condition interactions for IL-12p70 ( $P = 0.017$ ; Figure 4A), IL-1 $\beta$  ( $P = 0.020$ ; Figure 4B), and ghrelin ( $P = 0.017$ ; Figure 4C). We also found potentially interesting trends for IL-6 ( $P = 0.154$ ; Figure 5A), IL-8 ( $P = 0.284$ ; Figure 5B), and MCP-1 ( $P = 0.141$ ; Figure 5C). These effects were consistent with the pattern observed for serum endotoxin in that sporebased probiotic intervention was associated with a reduction in a given biomarker at post-supplementation compared to pre-supplementation and placebo.

**DISCUSSION** An a priori review of the existing literature[5,29], lead our team to speculate that there may be an ideal subject phenotype that was “responsive” to sporebased probiotic treatment. Thus, we designed and implemented a screening protocol for the present study to identify individuals who presented with postprandial endotoxemia at baseline, which may be a hallmark sign of intestinal permeability and “leaky gut” syndrome[14,15,22,23]. We believe our approach to subject selection increased the efficacy and applicability of our key findings. Within our “responder” population (who likely had a non-protective microbiome), we were able to demonstrate that 30-d of oral supplementation with a viable, spore-based probiotic was associated with a significant reduction in post-prandial endotoxin and triglycerides. Further, we found that several of our exploratory biomarkers were either significantly reduced (IL-12p70, IL-1 $\beta$ , and ghrelin) or trended toward reduction (IL-6, IL-8, and MCP-1) with sporebased probiotic supplementation. It is reasonable to speculate that the spore-based probiotic supplement may have exerted its effect by altering the gut microbial profile, altering intestinal permeability, or a combination of the two effects. The present study was designed to assess systemic changes rather than focus on intestinal measures that are invasive or impossible to make accurately in human subjects. The reductions observed

Pre 3-h post 5-h post Pre 3-h post 5-h post

35

30

25

20

15

10

5

0

IL-12-p70 (pg/mL)

Pre 3-h post 5-h post Pre 3-h post 5-h post

2.5

2.0

1.5

1.0  
 0.5  
 0.0  
 IL-1 $\beta$  (pg/mL)  
 Pre 3-h post 5-h post Pre 3-h post 5-h post  
 12  
 10  
 8  
 6  
 4  
 2  
 0

Ghrelin (pg/mL)

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Figure 4 Serum IL-12p70 (A), IL-1 $\beta$  (B), and ghrelin (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects consumed an oral probiotic supplement for 30-d and the experimental meal challenge was completed at baseline and following the 30-d supplementation period. Probiotic responses were compared to placebo. IL: Interleukin.

A B

C

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in the present study with spore-based probiotic supplementation were consistent with a transient reduction in chronic disease risk. It is also important to note that the reported changes were observed while the college-aged subjects continued to lead their habitual life with no directed modification. They continued to be exposed to many of the stressors that are known to negatively affect gut permeability in college-aged individuals (i.e., consumption of microwaved and other processed food, fast foods, soft drinks with their excess of sugars, including artificial sugars, colorings and flavorings, energy drinks, alcohol consumption, lack of sleep, exam anxiety, etc.). Previous authors consistently speculate that the onset and progression of chronic disease results from the accumulation of transient changes in ones' health that result from lifestyle choices[16,18,19,22,28,30-32].

Unfortunately, the current literature has yet to define the quantity of transient change that must be accumulated to cause disease onset. Instead, previous studies have attempted to use lifestyle modifications (i.e., nutrition, physical activity, etc.) to minimize negative changes in health. One such problem, especially in western cultures, is the wide accessibility to high-fat, high-calorie meals, creating an environment where excessive, low-quality nutritional

habits are the norm. In these diets elevated post-prandial endotoxin and triglyceride are consistently reported as problematic changes. Our observed baseline responses mirror previous reports[22,31-33]. Recently a review article touted the potential of probiotic

supplementation to prevent metabolic or dietary endotoxemia[24], but to our knowledge no published study has yet to demonstrate this outcome. Thus, our finding of a 42% reduction in metabolic endotoxemia is novel and unique. Further interpretation of our finding does reveal a potentially interesting effect, while 30-d of supplementation reduced metabolic endotoxemia by 42%, it did not completely prevent metabolic endotoxemia. It is plausible to speculate that a longer period of supplementation may result in greater reductions in metabolic endotoxemia. Cani et al[14,15] previously reported in rodents, that the only viable method to “reprogram” the gut microbial response was to initially treat animals with a broadspectrum antibiotic. For obvious ethical reasons treating human subjects with antibiotics is likely not a viable experimental design consideration, but perhaps the same effect could be achieved with a longer period of probiotic supplementation. In addition to probiotic effects, we also observed an interesting response in placebo subjects. Specifically, the placebo subjects presented

Pre 3-h post 5-h post Pre 3-h post 5-h post

16  
14  
12  
10  
8  
6  
4  
2  
0

IL-6 (pg/mL)

Pre 3-h post 5-h post Pre 3-h post 5-h post

10 9 8 7 6 5 4 3 2 1 0 IL-8 (pg/mL)

Pre 3-h post 5-h post Pre 3-h post 5-h post

250  
200  
150  
100  
50  
0

Ghrelin (pg/mL)

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Spore-based probiotic Placebo

Figure 5 Serum IL-6 (A), IL-8 (B), and MCP-1 (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects consumed an oral probiotic supplement for 30-d and the experimental meal challenge was completed at baseline and following the 30-d supplementation period. Probiotic responses were compared to placebo. While effects did not reach statistical significance, trends are consistent with other variables that did significant change (Figures 2 and 3). IL: Interleukin.

A B

C

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with an even greater metabolic endotoxemia response following a 30-d period. We do not believe that this observation is due to the experimental treatment, but is rather likely due to a diurnal fluctuation in metabolic endotoxemia responses. Thus, placebo subjects trended toward increased metabolic endotoxemia, while probiotic intervention reversed that effect. Since the present 30-d probiotic intervention did not completely prevent metabolic endotoxemia, it is reasonable to speculate that an intervention longer than 30-d may be necessary to completely prevent metabolic endotoxemia. We have previously demonstrated that the consumption of a high-fat meal causes transient biological changes that were consistent with a transient increase in risk of atherosclerosis[16,18,21,32]. These changes combined with a post-prandial increase in serum triglycerides creates a milieu that favors foam cell formation and the development of atherosclerotic plaques[19,31,33,34]. In the present study, the baseline post-prandial meal response presented outcomes that were consistent with published data from our laboratory and others[16,18,19,22,31-33,35]. Thus, the present study presented an opportunity to assess if a probiotic intervention would change disease risk biomarkers in a similar manner as endotoxin and triglycerides. We found significance across the entire meal combined between conditions, but were unable to tease apart specific time point differences. A post hoc sample size analysis revealed that we would have needed to enroll approximately 20 “responder” subjects in each group to delineate specific time point changes for biomarkers. Regardless, we found significant reductions in IL-12p70, IL-1 $\beta$ , and ghrelin. Previous research has indicated that obese subjects do not have as great of a post-prandial suppression ghrelin than normal weight subjects[36]. The authors do not explain the nature of the change, but given the observations of the present study, it is reasonable to speculate that obesity status may very well effect the gut microbiome[36]. It is plausible that in the present study, without changing body weight, we were able to create the microbiome of a normal weight individual thus restoring normal post-prandial ghrelin responses. Given the pro-inflammatory actions of IL-1 $\beta$ , the observed reduction with probiotic supplementation was consistent with reductions in post-prandial systemic inflammation. Reduced ghrelin may be indicative of better post-prandial hunger/satiety control with probiotic. IL-12p70 has a variety of metabolic actions, the chief action in the present study is the ability to modulate the release of TNF- $\alpha$  or related inflammatory cytokines following antigenic challenge[37,38]. In the case of the present study, reduced IL-12p70 with probiotic supplementation may reflect a reduction in systemic inflammatory capacity. In addition to the biomarkers that reached significance, we also found similar numerical trends for IL-6, IL-8, and MCP-1, which are all released by adipose tissues and commonly elevated in obese individuals[27,31,39]. The biomarkers observed to change in the present study following the probiotic intervention are involved in the accumulation of systemic inflammation[38,40-43]. The existing literature has linked elevated systemic inflammation to the pathophysiology of cardiovascular and metabolic diseases, thus even a transient reduction in systemic inflammation biomarkers may be associated with reduced disease risk[2,24]. The biomarkers measured in the present study are most often measured in the context of long-term weight loss (> 12 wk) interventions. In those weight loss models, it

can take up to 16-wk to reduce body weight enough that biomarkers change. It is interesting that we demonstrated similar reductions in inflammatory biomarkers in 1/4 the time, but also in the absence of weight loss. We have presented novel results concerning the ability of probiotic supplementation to elicit transient effects. In summary, the key findings of the present study demonstrate that 30-d of spore-based probiotic supplementation resulted in a blunting of dietary endotoxin, triglycerides, and potentially systemic inflammation. To our knowledge, the present study is the first to report that a short-term spore-based probiotic intervention altered dietary endotoxemia in human subjects, although the effect has been widely reported in mice [1,14]. Due to limitations associated with using human subjects, it was not possible to directly measure gut permeability in the present study. Despite this, it is reasonable to speculate that the underlying cause of the observed reductions in post-prandial endotoxemia may be due to changes in the gut microbiome, gut permeability, or a combination of the two. Future research is needed to determine if a longer course of treatment with a spore-based probiotic results in additional health improvements.

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**Background:** It has been suggested that gut microbiota is altered in Type 2 Diabetes Mellitus (T2DM) patients.

**Objective:** This study was to evaluate the effect of the prebiotic xylooligosaccharide (XOS) on the gut microbiota in both healthy and prediabetic (Pre-DM) subjects, as well as impaired glucose tolerance (IGT) in Pre-DM.

**Subjects/Methods:** Pre-DM (n = 13) or healthy (n = 16) subjects were randomized to receive 2 g/day XOS or placebo for 8-weeks. In Pre-DM subjects, body composition and oral glucose tolerance test (OGTT) was done at baseline and week 8. Stool from Pre-DM and healthy subjects at baseline and week 8 was analyzed for gut microbiota characterization using Illumina MiSeq sequencing.

**Results:** We identified 40 Pre-DM associated bacterial taxa. Among them, the abundance of the genera *Enterorhabdus*, *Howardella*, and *Slackia* was higher in Pre-DM. XOS significantly decreased or reversed the increase in abundance of *Howardella*, *Enterorhabdus*, and *Slackia* observed in healthy or Pre-DM subjects. Abundance of the species *Blautia hydrogenotrophica* was lower in pre-DM subjects, while XOS increased its abundance. In Pre-DM, XOS showed a tendency to reduce OGTT 2-h insulin levels (P = 0.13), but had no effect on body composition, HOMA-IR, serum glucose, triglyceride, satiety hormones, and TNF $\alpha$ .

**Conclusion:** This is the first clinical observation of modifications of the gut microbiota by XOS in both healthy and Pre-DM subjects in a pilot study. Prebiotic XOS may be beneficial in reversing changes in the gut microbiota during the development of diabetes.

Clinical trial registration: NCT01944904 (<https://clinicaltrials.gov/ct2/show/NCT01944904>).

Keywords: xylooligosaccharide, prediabetic, diabetes, gut, microbiota

Abbreviations: XOS, xylooligosaccharide; Pre-DM, prediabetic; BMI, body mass index; T2DM, Type 2 Diabetes Mellitus; FOS, fructooligosaccharides; GOS, galactooligosaccharides; OGTT, oral glucose tolerance test OGTT.

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### Introduction

Increasing evidence indicates that changes in gut microbiota composition might contribute to the development of metabolic disorders such as obesity and T2DM (Qin et al., 2012). Several mechanisms have been proposed regarding how gut bacteria could facilitate the pathogenesis of T2DM (Shen et al., 2013). Studies suggest that gut bacteria influence wholebody metabolism through regulation of the host's immune response, energy extraction and utilization, intestinal glucose absorption, and lipid metabolism (Musso et al., 2011). The central feature of obesity and T2DM is insulin resistance, potentially caused by low-grade inflammation resulting from nutrient excess and leading to endoplasmic reticulum (ER) stress. More and more research has shown that gut microbiota is another important factor for this low-grade inflammation (Chassaing and Gewirtz, 2014). The Bacteroidetes/Firmicutes ratio is associated with increased plasma glucose concentrations and a decrease in butyrate-producing bacteria in T2DM patients (Larsen et al., 2010; Shen et al., 2013). Recent studies in mice have demonstrated that an increase in the abundance of Bifidobacteria and Akkermansia muciniphila attenuated high fat diet-induced metabolic complications (Everard et al., 2013). Pre-DM refers to the intermediate stage between normoglycemia and overt diabetes mellitus. Pre-DM is characterized by glucose dysregulation and a higher risk of developing T2DM and other associated complications (Portero McLellan et al., 2014). However, not all individuals with Pre-DM progress to overt T2DM. With changes in lifestyle and diet, the progression of Pre-DM to T2DM can be prevented or delayed (Portero McLellan et al., 2014). Given the significant involvement of certain gut bacteria in host metabolism (Shen et al., 2013), therapeutic manipulation of the gut microbiota has been proposed for both individuals with T2DM and in those at risk of developing the condition. Prebiotics are highly effective and important for many applications in medicine. They are not digestible and do not contribute to human nourishment, but rather exert a profound effect on the human gut microbiota (International Scientific Association for Probiotics and Prebiotics, 2004). Prebiotic-induced modulation of gut microbiota has been developed and widely used (Everard et al., 2011). The principal effect of prebiotics on the human gut microbiota is to stimulate the growth of the Bifidobacterium and Lactobacillus genera (Marotti et al., 2012). Prebiotic treatment is known to modulate host gene expression and metabolism as well (Everard et al., 2011). Dietary intervention using prebiotic inulin or oligofructose (FOS) alters the gut microflora composition by promoting the growth of beneficial bacteria such as Bifidobacterium, Lactobacillus, and A. muciniphila (Rossi et al., 2005; Choi and Shin, 2006). However, the mechanisms associating prebiotics and its beneficial effects have yet to be fully understood. Further studies are needed to examine the precise physiological roles of prebiotics on human bowel flora and in host immune function. Xylooligosaccharide (XOS) is a recent prebiotic that can be incorporated into many food products (Aachary and Prapulla, 2011). Our lab previously reported that XOS, at the dose of

2.8g/day, was well tolerated, and modified the gut bacterial composition in healthy people (Finegold et al., 2014). A consideration of the gut microbiota in the context of health benefits of XOS in Pre-DM is especially relevant since recent research has indicated a critical role of gut microbiota in the development of T2DM (Qin et al., 2012). The present study was designed to determine the effect of XOS supplementation on the gut microbiota in healthy and pre-DM individuals. Since the ultimate objective of this research is to explore the potential effects of XOS in the prevention of progression of Pre-DM to T2DM, we also evaluated the effects of XOS in the management of IGT, body composition, and inflammatory marker in Pre-DM subjects.

#### Materials and Methods

**Subjects** This was a double-blind, randomized, placebo-controlled study with 34 subjects who were recruited based on inclusion and exclusion criteria. The study population consisted of 16 healthy subjects (placebo: n = 9; XOS: n = 7) and 13 Pre-DM subjects (placebo: n = 6; XOS: n = 7).

**Ethics** The study was carried out in accordance with the guidelines of the Office for Protection of Research Subjects of the University of California, Los Angeles and the Institutional Review Board of the VA Greater LA Health Center. All subjects provided written informed consent before the study began.

**Study Design** The enrollment criteria for healthy participants was fasting plasma glucose of 65–100 mg/dl, Participants in the Pre-DM study were selected based on the American Diabetes Association criteria for impaired fasting glucose (fasting plasma glucose of 100–125 mg/dl) and/or HgbA1c (5.7–6.4%) (American Diabetes Association, 2014). Over the span of 8 weeks, both healthy and Pre-DM subjects were randomly assigned to take daily a capsule supplement containing either 2 g XOS (2.8 grams of 70% XOS) or placebo. The XOS and placebo were provided by Life Bridge International (Riverside, CA). The XOS was manufactured by Shandong Longlive Bio-Technology Co., Ltd., China. The placebo capsules contained maltodextrin. The study consisted of three phases: a 2-week run-in phase, and an 8-week intervention phase.

**Stool Collection** A total of two stools were collected from each subject: at baseline and week 8 of the intervention periods. Each time the entire stool specimen was obtained. The specimen was placed in a large, ziplock freezer bag and all air was pushed out of the bag as the zip lock was closed. The specimen was delivered on ice to the UCLA Center for Human Nutrition within 24h of collection where it was immediately stored at  $-20^{\circ}\text{C}$ .

**Miseq Sequencing** DNA from stool was extracted using a commercial extraction system (QIAamp Stool DNA Extraction Kit, Qiagen, Valencia,

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CA). The quality of the DNA samples was confirmed using a Bio-Rad Experion system (Bio-Rad Laboratories, CA, USA). The 16S rRNA gene V4 variable region PCR primers 515/806 with barcode on the forward primer were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions:  $94^{\circ}\text{C}$  for 3min, followed by 28 cycles of  $94^{\circ}\text{C}$  for 30s,  $53^{\circ}\text{C}$  for 40s and  $72^{\circ}\text{C}$  for 1min, after which a final elongation step at  $72^{\circ}\text{C}$  for 5min was performed. After amplification, PCR products are checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using a

proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al., 2006).

**Body Composition** Body composition was measured using the Tanita-BC418 bioelectrical impedance analyzer (Tanita Corp., Japan).

**Glucose Tolerance Test** On the test days at baseline and week 8, Pre-DM subjects remained in a fasting state for 2.5h prior to the beginning of OGTT. The 75g glucose cola was administered immediately after the basal blood draw at 0min. Subsequent blood samples were taken 30, 60, and 120min afterwards. Serum samples were kept at  $-80^{\circ}\text{C}$ . The insulin resistance index assessed by the homeostasis model (HOMA-IR) was calculated as follows (Allard et al., 2003):  $(\text{fasting blood glucose [mmol/l]} \times \text{fasting plasma insulin [\mu U/ml]}) / 22.5$ .

**Blood Biochemical Analysis** Blood samples were collected and coded to protect patient confidentiality. Lipids, insulin, glucose, and satiety hormones were measured. Serum triglycerides were determined using an enzymatic method (Pointe Scientific, MI). Serum glucose was determined using Glucose Assay kit (Cayman Chemical Company, MI). Serum insulin, active GLP-1, leptin, pancreatic polypeptides (PP) and  $\text{TNF}\alpha$  were determined using the MILLIPLEX map kit (EMD Millipore, Billerica, MA) and data were captured and processed using Luminex 200 with xPonent software.

**Statistics** Demographic data at baseline were analyzed and presented as mean  $\pm$  SD and two sample t-tests were used for comparisons between the placebo and XOS groups. For analysis of serum variables, such as insulin, glucose, values of mean  $\pm$  SE at baseline and week 8 were presented for all groups, and two sample t-test was used to compare the two groups at baseline and 8 weeks. For DNA sequencing analyses, Wilcoxon rank sum test was utilized to evaluate the differences between study groups. All tests are two sided and all analyses were conducted using SAS 9.3 (Statistical Analysis System, Cary, NC, 2008) and R ([www.r-project.org](http://www.r-project.org)) software.

## Results

**Subjects** The study population consisted of 16 healthy participants (4 men and 12 women) aged between 21 and 49 years, and 13 Pre-DM participants (9 men and 4 women) aged between 30 and 63. Participants did not report any adverse effects or symptoms with the XOS intervention at 2g/day. Table 1 shows the baseline characteristics of the participants in the four groups. The composition of the fecal microbiome from all healthy participants and Pre-DM participants was analyzed by Miseq sequencing.

**Gut Microbial Composition Changes Related to Pre-DM** Miseq sequencing was used to compare the gut microbial composition of baseline samples from 16 healthy and 13 Pre-DM subjects. The abundance (percentage of total sequences) of 1 phylum, 1 class, 3 families, 13 genera, and 22 species, was significantly different between healthy and Pre-DM (Supplementary Table 1). Composition of phyla in healthy and Pre-DM groups is displayed in Figure 1A. The abundance of infectious and T2DM related phylum Synergistetes (Baumgartner et al., 2012; Qin et al., 2012) was significantly higher in Pre-DM compared with healthy subjects ( $P \leq 0.05$ ) (Figure 1A). In addition, 13 genera responded significantly in Pre-DM ( $P \leq 0.05$ ) (Figure 1B). The abundances of Allisonella, Cloacibacillus, Enterorhabdus, Howardella, Megamonas, and Slackia were significantly higher, while Adlercreutzia, Anaerococcus, Ethanolgenens, Gordonibacter, Lactococcus, Parasutterella,

and *Tissierella* were greatly reduced in Pre-DM compared with healthy subjects (Figure 1B).

TABLE 1 | Baseline characteristics of the study participants.

Characteristic	Healthy placebo (n = 9)	Healthy XOS (n = 7)	Pre-DM placebo (n = 6)	Pre-DM XOS (n = 7)
Age, year	31.9 ± 6.9	31.7 ± 9.3	44.8 ± 11.2	55.0 ± 6.2
Weight, kg	65.4 ± 14.0	69.9 ± 13.0	94.9 ± 15.9	97.4 ± 22.7
BMI, kg/m <sup>2</sup>	23.4 ± 3.2	25.6 ± 3.2	33.6 ± 7.2	32.2 ± 4.2

Values are presented as mean ± standard deviation (SD). For all characteristics, there were no significant differences between the placebo and XOS groups (all  $P > 0.05$ , based on independent sample t-tests). BMI, body mass index.

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FIGURE 1 | Comparison of gut microbiota composition between healthy (n = 16) and Pre-DM (n = 13) subjects. (A) Pie charts depict mean abundance (% of total) of the indicated phyla. (B) Bar graph of

genera shows significant differences in abundance between healthy and Pre-DM subjects. Values are presented as mean ± standard error (SE) \* $P \leq 0.05$ .

Effects of XOS Supplementation on the Gut Microbiota in Healthy and Pre-DM Subjects The overall changes of bacterial composition with 8-week XOS intervention were assessed at the phylum and genus levels (Figures 2, 3). Significant XOS-induced changes were found for 1 phyla, 3 classes, 7 families, 23 genera, and 25 species in healthy subjects (Figure 2 and Supplementary Table 2), and 3 classes, 1 families, 7 genera, and 17 species in PreDM subjects (Figure 3 and Supplementary Table 3). The mean abundance of indicated phyla of healthy subjects in placebo and XOS groups at baseline and week 8 are displayed in Figure 2A. The phylum Firmicutes showed a 20% increase in abundance over 8 weeks in placebo groups of healthy subjects, while XOS intervention significantly reversed this increase ( $P \leq 0.05$ ) (Figure 2A). The mean abundance of Verrucomicrobia increased in XOS groups of healthy subjects as well. Among genera with an average abundance >1% in at least one group, six were significantly regulated by XOS. An increase of infectious disease related *Streptococcus* and *Subdoligranulum* in placebo groups was largely inhibited by XOS in healthy subjects (Figure 2B). The 8-week XOS intervention did not induce significant changes of gut microbiota at the phylum level in Pre-DM subjects (Figure 3A). At the genus level, *Blautia*, *Anaerotruncus*, *Dialister*, and *Oscillospira* were four abundant genera identified with significant XOS-induced changes in Pre-DM subjects. XOS diminished or reversed the magnitude of population decline in all four genera (Figure 3B).

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FIGURE 2 | Effects of XOS supplementation on gut microbiota in healthy subjects (n = 16).

(A) Pie charts display the mean abundance of indicated phyla of healthy subjects receiving placebo (n = 9) and XOS (n = 7)

at baseline and week 8. (B) Bar graph of genera shows significant difference in abundance between placebo and XOS groups. Values are presented as mean ± standard error (SE) \* $P \leq 0.05$ .

XOS Supplementation Reversed Gut Bacterial Alterations Associated with Pre-DM Of the 40 Pre-DM associated bacterial taxa (Supplementary Table 1) identified in this study, the abundances of the *Enterorhabdus*, *Howardella*, and *Slackia* genera were elevated in Pre-DM. The 8-week XOS intervention significantly diminished or reversed the abundance

increase of *Howardella* and *Slackia* observed in the placebo group of healthy subjects, as well as *Enterorhabdus* in Pre-DM subjects (Figures 4A–C). *B. hydrogenotrophica* was less abundant in Pre-DM subjects (Supplementary Table 1), but XOS intervention significantly reversed the decrease in *B.*

*hydrogenotrophica* abundance observed in the placebo group of Pre-DM subjects ( $P \leq 0.05$ ) (Figure 4D).

**Effects of XOS on Body Composition, Metabolic, and Immunological Markers in Pre-DM Subjects** In Pre-DM subjects, body composition, blood tests, and oral glucose tolerance tests (OGTT) were done at baseline and after 8 weeks of XOS intervention. Body weight and indexes of overall adiposity such as BMI, % fat, and % trunk fat were not changed by 8-week XOS intervention (Figure 5). Despite significant inter-individual variations in insulin responses

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FIGURE 3 | Effects of XOS supplementation on gut microbiota in Pre-DM subjects ( $n = 13$ ).

(A) Pie charts display the mean abundance of indicated phyla of Pre-DM subjects receiving placebo ( $n = 6$ ) and XOS

( $n = 7$ ) at baseline and week 8. (B) Bar graph of genera shows significant difference in abundance between placebo and XOS groups. Values are presented as mean  $\pm$  standard error (SE) \* $P \leq 0.05$ .

among Pre-DM subjects, OGTT 2-h insulin response showed a tendency to decrease with XOS intervention in Pre-DM ( $P = 0.13$ ) (Figure 6A). No significant XOS-related differences were observed in serum glucose, HOMA-IR, active GLP-1, triglycerides, leptin, PP, or the inflammatory marker  $TNF\alpha$  (Figures 6B–H).

#### Discussion

Emerging evidence suggests that metabolic disorders including T2DM are associated with a pro-inflammatory state secondary

to dysbiosis of gut bacterial flora (Larsen et al., 2010; Esteve et al., 2011; Musso et al., 2011). In addition, literature has documented the translocation of gut bacteria to blood and tissues in T2DM, and probiotic *Bifidobacterium* treatment prevents bacterial translocation and protects against T2DM (Cani et al., 2007; Amar et al., 2011). Together, these findings suggest that gut bacteria are an important modifier of T2DM. The effects of

oligosaccharides including XOS, FOS, and galactooligosaccharides (GOS) in the treatment of T2DM have gained interest. However, studies have shown inconsistent results. In T2DM patients, Yamashita et al. demonstrated that

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FIGURE 4 | XOS selectively regulated some of the Pre-DM associated bacterial taxa in healthy subjects ( $n = 16$ ) or Pre-DM subjects ( $n = 13$ ) during 8 weeks. The abundances of Pre-DM associated *Howardella* (A), *Slackia* (B), and *Enterorhabdus* (C) were greatly reduced by XOS in healthy and Pre-DM subjects, respectively. (D) The abundance of healthy associated *Blautia hydrogenotrophica* was enhanced by XOS in healthy and Pre-DM subjects. Values are presented as mean  $\pm$  standard error (SE) \* $P \leq 0.05$ .

FIGURE 5 | Body weight (A), BMI (B), % Fat (C), and % Trunk fat (D) in Pre-DM subjects at baseline and after 8 weeks placebo ( $n = 6$ ) or XOS ( $n = 7$ ) treatment. Data are means  $\pm$  standard errors (SE).

FOS at a dose of 8g per day for 14 days resulted in a reduction of serum glucose, while Alles et al. showed that daily consumption of FOS at 15g for 20 days had no effect on serum glucose level (Yamashita et al., 1984; Alles et al., 1999). Chan et al. showed that 4g per day of XOS for 8 weeks was effective in reducing blood glucose and lipids in Taiwan T2DM patients (Sheu et al., 2008), and for 21 days benefited intestinal health and increased of Bifidobacteria in elderly subjects (Chung et al., 2007). Another study reported that both XOS and FOS dietary intervention reduced hyperglycaemia in diabetic rats (Gobinath et al., 2010). In addition, we found that 2 g per day of XOS for 8 weeks increased the Bifidobacteria abundance in healthy Americans without any gastrointestinal side effects (Finegold et al., 2014). Overall, a significant number of studies have shown oligosaccharides to be an effective option for lowering blood sugar in T2DM as well as improving intestinal health.

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FIGURE 6 | Mean of parameters with SE at baseline and 8 weeks were compared between placebo- (n = 6) and XOS-treated (n = 7) group in Pre-DM subjects during the 120-min OGTT test. (A) Serum

Insulin. (B) Serum glucose. (C) HOMA-IR. (D) Serum active GLP-1. (E) Serum triglyceride. (F) Serum pancreatic polypeptides. (G) Serum leptin. (H) Serum TNF  $\alpha$ . Values are presented as mean  $\pm$  standard error (SE).

In the present study, both healthy and Pre-DM subjects were given 2 g per day of XOS for 8 weeks. Miseq sequencing was used to evaluate the potential of XOS in preventing the dysbiosis of gut microbiota during the development of T2DM. We found that XOS had a clear impact on gut microbiota in both healthy and Pre-DM groups, and resulted in dramatic shifts of several bacterial taxa associated with Pre-DM. Among them, *Dialister* spp. and *Slackia* are pro-inflammatory (Rocas and Siqueira, 2006; Kim et al., 2010), and were greatly reduced by XOS. Additionally, T2DM associated lactic acid bacteria *Enterococcus*,

*Streptococcus*, and *Lactobacillus* (Remely et al., 2013) were also greatly reduced by XOS. The inhibitory effect of XOS on other opportunistic pathogens, such as *Clostridia*, *Streptococcaceae*, and *Subdoligranulum*, further supports that XOS can potentially promote an optimal gut microbiota profile, and consequently reduce the risk of T2DM. Miseq sequencing data also revealed that gut microbial composition of healthy subjects at different taxonomic levels was different from Pre-DM subjects. Some of our findings are consistent with previous T2DM studies of gut microflora

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(Qin et al., 2012; Zhang et al., 2013; Finegold et al., 2014), substantiating the significant association of these changes with the progression of T2DM (Supplementary Table 1). Studies showed that the *Megamonas* OTU was most enriched in PreDM, compared to healthy or T2DM individuals (Qin et al., 2012; Zhang et al., 2013). The abundance of *Megamonas* in our study was 200-fold higher in Pre-DM than healthy subjects. We also found that the abundance of the phylum *Synergistetes* in PreDM was about 50 fold higher compared with healthy subjects. The *Synergistetes* appear to be more numerous in individuals with oral-related diseases as well as gut and soft tissue infections (Vartoukian et al., 2007). Another two infectious or metabolic disease related bacteria *Eubacteriaceae*

(Plieskatt et al., 2013) and *Slackia* (Kim et al., 2010), were more abundant in Pre-DM individuals. The Pre-DM associated enrichment of infectious bacteria and the appearance of oral bacteria in the gut suggest that the host immune system may lose control over these opportunistic pathogens during the development of T2DM. Both animal and clinical studies have shown that XOS supplementation greatly increases the *Bifidobacterium* population (Campbell et al., 1997; Chung et al., 2007; Finegold et al., 2014). We previously showed that an increase of *Bifidobacterium* abundance was detectable only with an in vitro culture method, and not pyrosequencing (Finegold et al., 2014). Using Miseq sequencing alone, the abundances of the *Bifidobacterium* genus, as well as the *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium adolescentis* species were not significantly increased by XOS in healthy subjects (Supplementary Table 4). However, we found that XOS largely inhibited bacterial taxa related to infectious and metabolic disease, such as family Streptococcaceae, class Clostridia, and genera *Subdoligranulum*, *Gordonibacter*, and *Streptococcus* in healthy subjects (Supplementary Table 1). Furthermore, in healthy subjects, XOS greatly reduced the abundance of bacteria related to obesity or T2DM, including phylum Firmicutes and genera *Subdoligranulum* and *Bacilli* (Remely et al., 2013; Zhang et al., 2013). In Pre-DM subjects, XOS diminished or reversed the magnitude of population decline in about 70% bacterial taxa identified with a significant change from its baseline levels between treatment groups (Supplementary Table 2). The family Veillonellaceae and genera *Oscillospira* and *Dialister* exhibited population declines in the placebo group, but demonstrated large increases in abundance in the XOS group. Abnormally low levels of Veillonellaceae and *Dialister* have been described in autistic children (Kang et al., 2013) and patients of Crohn's disease (Joossens et al., 2011). Dietary whole grain intervention (Martinez et al., 2013) and corn fiber (Hooda et al., 2012) increased the *Dialister* and Veillonellaceae abundance. The genus *Oscillospira* has been associated with lean BMI (Tims et al., 2013). The inhibition of Firmicutes and increase of *Oscillospira* abundance suggest a potential role of XOS in weight control. To the best of our knowledge, this is the first clinical study evaluating the effects of daily treatment with 2 g of XOS on glucose tolerance and insulin resistance in Pre-DM adults. In our experience, a dose of 2g does not cause any gastrointestinal side effects (Finegold et al., 2014). Eight weeks of XOS supplementation tended to increase insulin sensitivity by lowering OGTT 2-h insulin response ( $P = 0.11$ ), while no significant improvement of Pre-DM subjects' metabolic situation was observed, using the parameters of body composition, serum glucose, triglyceride, satiety hormones and inflammation marker TNF $\alpha$ . The TNF $\alpha$  levels ( $4.91 \pm 1.85$  pg/ml) of Pre-DM subjects in our study are normal, slightly lower than the reported TNF $\alpha$  (~15–20pg/ml) of eastern Indian Pre-DM population (Dutta et al., 2013) and much lower than T2DM (range from 87 to 112pg/ml) (Goyal et al., 2012). Since we enrolled pre-DM subjects with impaired glucose tolerance (IGT) and without any other medical conditions we possibly did not observe elevated TNF $\alpha$  level. Pre-DM is a dynamic intermediate stage in the progression to T2DM, therefore it is very likely, subjects that met the selection criteria for Pre-DM are at different stages even though they are all classified as Pre-DM. Besides, studies also suggest a connection between TNF $\alpha$  gene polymorphism, its blood levels and the tendency to progression from Pre-DM to T2DM (Dutta et al., 2013). Therefore, more studies are needed to improve our understanding of the relationship between TNF $\alpha$ , Pre-DM staging, and T2DM progression. Our results do not agree with Chan

et al. study in T2DM. However, this discrepancy could be explained by difference in XOS dose, study population and disease stages. It is possible that 2 g per day of XOS may have been too low to induce a difference in glucose tolerance. However, we observed a trend of increased insulin sensitivity by lowering OGTT 2-h insulin response at this dosage. Pre-DM is a strong risk factor for the development of T2DM, and the regulation of glucose metabolism and insulin sensitivity could be really dynamic during this stage. We think future clinical study with large sample size will be needed to confirm the benefits of XOS in Pre-DM. In conclusion, XOS significantly modified gut microbiota in both healthy and Pre-DM subjects, and resulted in dramatic shifts of 4 bacterial taxa associated with Pre-DM. Future studies with larger sample size are needed to study the metabolic impact of XOS and understand the connection between XOS-mediated gut microbiota changes and the pathogenesis of T2DM.

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ZL and DH designed research and had primary responsibility for final content. SH, JY, MH, HM, JH, PS, and G. Thames conducted research. SF, CT, and JY analyzed the data. JY wrote paper.

### A Mixture of *trans*-Galactooligosaccharides Reduces Markers of Metabolic Syndrome and Modulates the Fecal Microbiota and Immune Function of Overweight Adults 1–3

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#### Abstract

Metabolic syndrome is a set of disorders that increases the risk of developing cardiovascular disease. The gut microbiota is altered toward a less beneficial composition in overweight adults and this change can be accompanied by inflammation. Prebiotics such as galactooligosaccharides can positively modify the gut microbiota and immune system; some may also reduce blood lipids. We assessed the effect of a galactooligosaccharide mixture [Bi2muno (B-GOS)] on markers of metabolic syndrome, gut microbiota, and immune function in 45 overweight adults with 3 risk factors associated with metabolic syndrome in a double-blind, randomized, placebo (maltodextrin)-controlled, crossover study (with a 4-wk wash-out period between interventions). Whole blood, saliva, feces, and anthropometric measurements were taken at the beginning, wk 6, and end of each 12-wk intervention period. Predominant groups of fecal bacteria were quantified and full blood count, markers of inflammation and lipid metabolism, insulin, and glucose were measured. B-GOS increased the number of fecal bifidobacteria at the expense of less desirable groups of bacteria. Increases in fecal secretory IgA and decreases in fecal calprotectin, plasma C-reactive protein, insulin, total cholesterol (TC), TG, and the TC:HDL cholesterol ratio were also observed. Administration of B-GOS to overweight adults resulted in positive effects on

the composition of the gut microbiota, the immune response, and insulin, TC, and TG concentrations. B-GOS may be a useful candidate for the enhancement of gastrointestinal health, immune function, and the reduction of metabolic syndrome risk factors in overweight adults. *J. Nutr.* 143: 324–331, 2013.

#### Introduction

Metabolic syndrome is a combination of disorders, such as glucose intolerance, central obesity, dyslipidemia, and hypertension, which increases the risk of developing cardiovascular disease, type 2 diabetes, and cancer (1). The development of metabolic syndrome is a complex process that involves genetic, environmental, and dietary factors [e.g., long-term stress, positive energy balance (excessive energy intake and low physical activity), diets comprising high fat and sugar and low micronutrients, disruption of chronobiology] and is associated with pathways that connect metabolism with the immune system and vice versa. Individuals affected by metabolic syndrome do not all present the same combination of disorders. Although excessive weight can sometimes be regarded as benign (2), it is generally accepted as being an important factor in metabolic syndrome. Regardless of an individual's weight, the chronic low-grade inflammatory condition that accompanies metabolic syndrome has been implicated as a major factor in both the onset of the syndrome and its associated pathophysiological consequences (3). Recent studies have shown that the gut microbiota varies in both its composition (e.g., reduced levels of bifidobacteria and increased levels of less desirable bacteria) and its metabolic activity in obese individuals compared with lean individuals (4–6). It seems that the “obese microbiota” can modulate host energy homeostasis and adiposity through a number of different mechanisms, including harvesting energy from food (7), LPS-induced chronic inflammation (8), modulation of tissue fatty acid composition (9), and gut-derived peptide secretion (10). Modulation of the gut microbiota by dietary means is the basis for the probiotic (11) and prebiotic (12) concepts. The majority of scientific data on prebiotic effects comes from studies with either inulin-type fructooligosaccharides (FOS) (7) or galactooligosaccharides (GOS) (12). This study was supported by a grant from Clasado Ltd. 2 Author disclosures: J. Vulevic had no conflicts of interest at the time of the study, nor during the analyses of samples and data, but is currently employed by Clasado Research Services, Ltd. A. Juric and G. Tzortzis are employed by Clasado Research Services, Ltd. G.R. Gibson, no conflicts of interest. 3 This trial was registered at [clinicaltrials.gov](http://clinicaltrials.gov) as NCT01004120. 6 Present address: Clasado Research Services Ltd, The University of Reading, Science and Technology Center, Reading, UK. \* To whom correspondence should be addressed. E-mail: [jvulevic@yahoo.com](mailto:jvulevic@yahoo.com). 7 Abbreviations used: BP, blood pressure; B-GOS, Bifidobacterium-GOS; FBC, full blood count; FISH, fluorescence in situ hybridization; FOS, fructooligosaccharide; G-CSF, granulocytes colony-stimulating factor; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; NDO, nondigestible oligosaccharide; sIgA, secretory IgA; TC, total cholesterol.

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at KAINAN UNIVERSITY on April 9, 2015 [jn.nutrition.org](http://jn.nutrition.org) Downloaded from galactooligosaccharides. The capacity of these prebiotics to selectively stimulate the growth of bifidobacteria, and in some cases lactobacilli, and elicit a significant change in the overall composition of the gut microbiota has been repeatedly demonstrated (13). With regard to obesity and related metabolic disorders, the majority of

available data related to prebiotics comes from animal models and dietary supplementation with FOS. These studies suggest that prebiotics are able to regulate food intake and weight gain, glucose homeostasis, dyslipidemia, steatosis, and hypertension (14). However, definitive evidence of an effect of prebiotics in obese humans is scarce and no study to our knowledge has looked at the effects of prebiotics on the gut microbiota, immune response, and markers of metabolic syndrome together. Furthermore, the effects of galactooligosaccharide supplementation in overweight individuals with respect to weight management, modulation of the gut microbiota, or metabolic syndrome have not been reported thus far, to our knowledge. In the present study, we investigated the effect of administering Bi2muno (B-GOS) on the fecal microbiota and on markers of metabolic syndrome and immune function in overweight adults in a double-blind, randomized, placebocontrolled, crossover study.

#### Subjects and Methods

**Materials.** Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich or BDH Chemicals. Fluorescent, Cy3labeled probes for fluorescence in situ hybridization (FISH), and cell culture media and supplements were obtained from Sigma-Aldrich.

**Subjects and study design.** More than 200 overweight volunteers predisposed to the development of metabolic syndrome and its associated increased risk of cardiovascular disease were invited for screening. From this pool of volunteers, written informed consent was obtained from 48 subjects who presented  $\geq 3$  risk factors for metabolic syndrome (15) [i.e., fasting glucose ( $>5.6$  mmol/L), high blood pressure (BP), dyslipidemia [low HDL cholesterol (HDL-C) ( $<1$  mmol/L), high TG ( $>1.3$  mmol/L), and large waist circumference ( $>94$  cm in men,  $>80$  cm in women)]. Further criteria for inclusion in the study were that subjects: were between 18 and 65 y of age; had a BMI  $>25$  kg/m<sup>2</sup>; had not had a myocardial infarction/stroke or cancer in the past 12 mo; were not diabetic (diagnosed or fasting glucose  $>7$  mmol/L) or suffering from other endocrine disorders; did not suffer from chronic coronary, renal, bowel disease/gastrointestinal disorders or have a history of cholestatic liver or pancreatitis; were not receiving drug treatment for hyperlipidemia, hypertension, inflammation, or hypercoagulation or using drugs that affected intestinal motility or absorption; had no history of alcohol/ drug abuse; were not planning or currently on a weight-reducing regime; were not taking any dietary antioxidant or other phytochemical, prebiotic, or probiotic supplements; were not pregnant or lactating; had not taken antibiotics for 1 mo prior to the start of the trial; were not anemic (hemoglobin: men  $>140$  g/L; women  $>115$  g/L); and did not smoke. Of the 48 subjects, 3 volunteers were withdrawn from the study after completing the second visit, because they were reluctant to fully comply with the study protocol. The trial protocol was reviewed and approved by The University of Reading Research Ethics Committee. Volunteers were randomly assigned to 1 of 2 groups: one started the trial with the placebo (maltodextrin) and the other with B-GOS, with both compounds provided in powder form (5.5 g/d) and supplied by Clasado. Subjects were asked to reconstitute the contents of the sachets immediately before consumption by mixing the powder with water and to consume the product every day at approximately the same time. They consumed the products for 12 wk, followed by a 4-wk washout period, before switching to the other intervention for the final 12 wk. Volunteers were required to visit The University of Reading on 7 separate occasions during this period. Their BP, BMI,

and waist circumference were recorded and bloods sampled at each visit (2–3 wk before the start, on d0, and after 6, 12, 16, 22, and 28 wk). During all visits (except 2–3 wk before the start), fecal samples were obtained and the use of any medication (including vitamin/mineral supplements) and adverse events recorded. In addition, habitual diet was assessed by prevalidated, 4-d, food diaries (2 weekend and 2 weekdays) before the start of the study and then twice during each intervention period. Information collected in the food diaries was analyzed using Dietplan 6 (Forestfield Software).

**Preparation and collection of fecal samples.** Fecal samples were collected, diluted, homogenized, and used to enumerate gut bacteria by FISH as previously described (16). A portion of each fecal sample was removed prior to dilution and used for secretory IgA (sIgA) and calprotectin analyses and to freeze-dry and determine the dry weight.

**Preparation and collection of whole blood.** Whole blood was collected in several different tubes depending on the analyses. EDTA-coated tubes were used for cytokine, CRP, insulin, and full blood count (FBC) analyses. FBC analyses were performed by the Pathology Department at the Royal Berkshire NHS Foundation Trust, Reading, UK, using a Sysmex XE-2100 automated hematology analyzer (Sysmex UK). Serum separation tubes-coated tubes were used for lipid analyses and fluoride oxalate tubes were used for glucose analysis.

**FISH.** The differences in bacterial populations were assessed using FISH analysis with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA as previously described (16). The probes used were Bif164 (17), Bac303 (18), Lab158 (19), Erec482 and His150 (20), Srb687 (21), Ato291 (22), Ecy1387 (23), Ehal469 (24), Bet42a (25), Prop853 (26), and Fprau645 (27), specific for *Bifidobacterium* spp., *Bacteroides* spp., *Lactobacillus/Enterococcus* spp., the *Clostridium coccooides/Eubacterium rectale* group, the *Clostridium histolyticum* group, *Desulfovibrio* spp., the *Atopobium* cluster, *Eubacterium cylindroides*, *Eubacterium hallii*, *b-Proteobacteria*, *Clostridium* cluster IX, and the *Faecalibacterium prausnitzii* cluster, respectively. For counts of total bacteria, the nucleic acid stain 4#, 6-diamidino-2-phenylindole was used. The number of cells obtained is expressed as per gram of dry weight feces.

**Measurement of cytokine production by whole blood cultures.** Blood (collected at the beginning and the end of each intervention) was diluted (1:10, v:v) in RPMI/Gln/Ab medium and plated onto 24-well plates in the presence of LPS (1 mg/L) and incubated at 37°C in an air: carbon dioxide (19:1) atmosphere for 24 h. Following incubation, the plates were centrifuged at 400 g for 5–6 min and the supernatants collected and frozen in aliquots (200 µL). Concentrations of cytokines [IL-6, IL-10, IL-8, TNF $\alpha$ , and granulocytes colony-stimulating factor (G-CSF)] were measured by ELISA using commercially available kits (Universal Biologicals Cambridge, except G-CSF, which was from R&D Systems Europe). The names of kits used and the catalog numbers are as follows: AssayMax Human IL-6 ELISA kit (EI1006–1), AssayMax Human IL-10 ELISA kit (EI3010–1), AssayMax Human IL-8 ELISA kit (EI1008–1), AssayMax Human TNF- $\alpha$  ELISA kit (ET2010–1), and Quantikine Human G-CSF (DCS50). Kits were used according to the manufacturers' instructions. The limits of detection for these assays were as follows: IL-6, <10 ng/L; IL-10, <100 ng/L; IL-8, <1 ng/L; TNF $\alpha$ , <10 ng/L; and G-CSF, <20 ng/L (data were supplied by the manufacturers of the kits).

**Analysis of calprotectin, CRP, insulin, and sIgA.** Fecal calprotectin, a marker of intestinal inflammation (PhiCal Calprotectin ELISA kit, K 6930, BioSupply UK), plasma CRP

(AssayMax Human CRP ELISA kit, EC 1001-1, Universal Biologicals Cambridge), and plasma insulin (Insulin ELISA kit, K 6219, Alere) as well as fecal and salivary sIgA (sIgA ELISA Kit, K 8870, Oxford Biosystems) were all measured by ELISA using commercially available kits and instructions provided by the manufacturers. The limits of detection for these assays were as follows: calprotectin, 2.915 mg/L; CRP, <100 ng/L; insulin, 3 pmol/L; and sIgA, 13.4 mg/L (data were supplied by manufacturers of the kits).

Analysis of plasma total cholesterol, HDL-C, LDL cholesterol, TG, and glucose. Whole blood was collected into appropriate tubes as

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described above and centrifuged for 10 min at 1600 g and 4°C. Plasma was removed and stored at 220°C until analysis. Samples were analyzed by using an ILab 600 biochemical analyzer and enzymatic colorimetric kits [IL Test TM Cholesterol, IL Test TM Triglycerides, and IL Test TM Glucose (0018250740), IL Test TM LDL-Cholesterol (0018255740), IL Test TM HDL-Cholesterol (0018256040), Instrumentation Laboratories].

Statistical analyses. All statistical tests were performed using SAS version 9.2. A mixed model was used allowing for the crossover nature of the study. The change from baseline for each measurement (variable) was used as a response and differences were sought between placebo and intervention having taken period effect and gender into consideration. Interaction terms were also considered in the modeling; the intervention period interaction was included as a test for carryover effects as well as interactions between intervention and gender. Where interaction terms and gender were not significant, they were removed from the model. Period and intervention terms remained in the model irrespective of the significance associated with them, because they define the crossover design of the study. Variables were split into primary (lipids, glucose, insulin, BP) and secondary (everything else). Due to the number of variables considered, P values were corrected for multiple testing using the Bonferroni correction. Following the Bonferroni adjustment, significance levels were set at  $P < 0.005$  for the primary variables and  $P < 0.0012$  for the secondary. Homogeneity of variance was tested using Levene's test. In the case of significantly different variances, Welch's test was used. Significant differences were not observed for any of the measured variables between the 2 groups at the start of each intervention period (baseline samples) and carry-over effect was not significant regardless of time, intervention, or variable. Values in the text are given as mean  $\pm$  SD.

## Results

**Participants.** A total of 45 volunteers (16 males, 29 females) completed the study.

Anthropometric and physiological data for the volunteers at the start of the trial are shown in Table 1. All participants had a BMI  $>25$  kg/m<sup>2</sup>; 98% of the participants had central obesity, 78% had plasma insulin concentrations  $>40$  pmol/L, 27% had plasma glucose concentrations  $>5.6$  mmol/L, 42% had BP  $>130/85$  mm Hg, 93% had plasma total cholesterol

(TC) concentrations  $>5.0$  mmol/L, 76% had low plasma HDL-C ( $<1.03$  mmol/L in men and  $<1.29$  mmol/L in women), and 40% had plasma TG concentrations  $>1.7$  mmol/L. Body weight and BP remained stable throughout the study for all participants regardless of intervention (data not shown). The habitual diets of participants at the start of the trial, as assessed by food diaries, provided 17.2  $\pm$  3.4%, 35.9  $\pm$  5.1%, and 42.3  $\pm$  6.6% of energy from protein, total fat, and carbohydrate, respectively. Energy and nutrient intake did not differ

between the 2 groups (i.e., placebo intervention first compared with B-GOS intervention first) at the start of the trial. Diet diaries indicated that the participants' habitual diets remained unchanged throughout the study (data not shown). In addition, FBC analyses, which included counts of white blood cells, platelets, neutrophils, lymphocytes, monocytes, eosinophils, and basophils, remained unchanged throughout the study for all participants regardless of intervention (data not shown).

**Populations of fecal bacteria.** The composition of the fecal microbiota of the overweight volunteers who participated in this study was determined by using FISH to enumerate 12 different bacterial groups of interest and total bacteria. There were no differences in populations of fecal bacteria in the 2 groups at the start of each intervention period (Table 2). The 2 dietary interventions had no significant effects on counts of total bacteria, *Lactobacillus/Enterococcus* spp., *Clostridium coccoides/Eubacterium rectale* group, *Atopobium* cluster, *E. cylindroides*, *E. hallii*, *b-Proteobacteria*, *Clostridium* cluster IX, and *F. prausnitzii* cluster during the study. However, after 6 wk and at the end of the study period, differences between interventions were observed with respect to the number of bifidobacteria, bacteroides, and *C. histolyticum* group bacteria detected ( $P < 0.0001$ ). That is, B-GOS increased the number of bifidobacteria in feces, whereas representation of the *Bacteroides* spp. and *C. histolyticum* group was lower (Table 2). The number of *Desulfovibrio* spp. tended to be lower during the B-GOS period than during the placebo period at 6 wk ( $P = 0.0018$ ) and was lower at the end of 12 wk ( $P < 0.0001$ ). In addition, the number

TABLE 1 Anthropometric and physiological data for the participants at the start of the trial

Characteristic Male (n = 16) Female (n = 29)

Age, y	42.8	6	12.1	46.4	6	11.8
BMI, kg/m <sup>2</sup>	30.7	6	5.3	32.1	6	6.3
Waist circumference, cm	104	6	11.0	99.2	6	14.5
Fasting insulin, pmol/L	77.7	6	33.6	65.0	6	29.2
Fasting glucose, mmol/L	5.5	6	0.8	5.2	6	0.6
Systolic BP, mm Hg	128	6	10.1	126	6	15.8
Diastolic BP, mm Hg	80.9	6	8.6	80.5	6	10.0
TC, mmol/L	6.6	6	1.2	6.1	6	1.3
HDL-C, mmol/L	1.2	6	0.2	1.5	6	0.3
TG, mmol/L	2.1	6	0.9	1.3	6	0.4

1 Values are mean  $\pm$  SD unless noted otherwise. BP, blood pressure; HDL-C, HDL cholesterol; TC, total cholesterol.

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of *b-Proteobacteria* found in fecal samples tended to be lower ( $P = 0.0019$ ) with B-GOS intervention than with placebo at 12 wk (Table 2).

**Cytokine production by whole blood cultures.** There were no significant effects (data not shown) of time or intervention on the production of the measured cytokines (G-CSF, IL-6, IL-10, IL-8, and TNF $\alpha$ ).

**Fecal calprotectin, plasma CRP, and fecal and salivary sIgA.** Changes in the concentrations of fecal calprotectin and plasma CRP at 6 and 12 wk for the placebo and B-GOS interventions from baseline are shown in Figure 1. The concentration of calprotectin was lower during the B-GOS intervention than during the placebo intervention both after 6 wk

and at the end of the 12-wk study ( $P < 0.0001$ ). The concentration of plasma CRP did not differ between the interventions at 6 wk; however, it was lower at the end of the 12 wk following B-GOS administration ( $P < 0.0012$ ). Neither intervention had any effect on sIgA concentrations in saliva (data not shown). However, the change in the concentration of fecal sIgA at the end of the 12 wk was significantly greater during the B-GOS period (808 6 176 mg/g dry weight

feces) than during the placebo period (2384 6 69 mg/g dry weight feces) ( $P < 0.0001$ ).

Plasma glucose and insulin. Glucose concentrations did not significantly differ at the beginning of each intervention and they remained unaffected regardless of intervention type or time during the trial period (Table 3). The plasma insulin concentration tended to be lower during the B-GOS period than during the placebo period after 6 wk ( $P = 0.008$ ) and was lower at the end of the 12-wk study period ( $P < 0.005$ ) (Table 3).

Plasma lipid profile. Plasma concentrations of HDL-C and LDL cholesterol (LDL-C) remained unchanged regardless of intervention type or time during the trial period (Table 3). In addition, both interventions had no effect on plasma concentration of TC after 6 wk, but by the end of the 12-wk study, B-GOS resulted in a

lower concentration compared with placebo ( $P < 0.001$ ) (Table 3). Plasma TG concentrations were lower ( $P < 0.0001$  at 6 wk and  $P < 0.0005$  at 12 wk) following administration of B-GOS compared with the placebo throughout the study period (Table 3). However, in the case of TG, the effect after 6 wk was significant ( $P < 0.005$ ) only in males (Fig. 2). The effect of gender was not significant by the end of the trial period (data not shown).

TABLE 2 Populations of bacteria in feces of overweight adults during 12-wk B-GOS and placebo interventions<sup>1</sup>

B-GOS	Placebo	Group	Baseline	2 wk	6 wk	12 wk	6 wk	12 wk																					
log <sub>10</sub> cells/g dry weight feces	Total bacteria	11.4	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5							
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5
6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0	2.11	4.6	0.2	11.5	6.0	2.11	4.6	0.2	11.4	6.0</				

adjustment for multiple testing, significance was set at  $P < 0.0012$ . Asterisks indicate different from placebo at that time: \* $P < 0.0001$ , \*\* $P < 0.0012$ . B-GOS, Bi2muno.

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The changes from baseline in the TC:HDL-C ratio at 6 and 12wk for placebo and B-GOS interventions are shown in Table 3. After 6 wk, the interventions had no significant effect. However, at the end of the 12 wk, B-GOS resulted in a lower TC:HDL-C ratio than the placebo ( $P < 0.0001$ ) (Table 3); this effect was more pronounced in males, as the intervention-gender interaction was significant at the end of each 12-wk intervention period ( $P < 0.005$ ) (Fig. 2).

#### Discussion

Excessive weight and diabetes are associated with a poor inflammatory status, leading to impaired insulin action and adipose-tissue plasticity (3). Although the origin of this inflammation is unclear, it seems that poor diet (high in fat, low in fiber) has direct pro-/anti-inflammatory effects, depending on its fatty acid composition and fiber content, and indirect effects through the gut microbiota and its ability to modulate metabolic endotoxemia, intestinal permeability, and inflammation. LPS, a component of the cell wall of Gram-negative bacteria that has been implicated in triggering low-grade inflammation (i.e., metabolic endotoxemia), is known to be increased in obese mice when they are fed a high-fat diet (27). Other studies in mice have shown that shifts of the gut microbiota toward a more beneficial composition (i.e., to a higher proportion of Gram-positive relative to Gram-negative bacteria) can decrease body weight, inhibit inflammation, and improve gut permeability (28,29). Beneficial colonic bacteria, namely lactobacilli and bifidobacteria, are Gram-positive bacteria that do not contain LPS within their cell membranes (30). Therefore, selective increases in numbers of these bacteria in overweight adults with metabolic syndrome can only be regarded as beneficial. This has been supported by several studies that have shown excessive weight gain induced following a high-fat diet (27,28,31,32) or genetic deletions (i.e., leptin-deficient models) (33) results in gut microbiota changes that reduce levels of bifidobacteria. Similar trends were observed in a study involving both genetic (apoA-I- deleted) and diet-induced murine models of metabolic syndrome, where bifidobacteria were reduced and *Desulfovibrio* spp. increased (34). In the latter study, diet was found to be a stronger contributor to microbial changes than the genetic alterations. In the only available human study comparing the microbiotas of lean and overweight adults, significantly fewer bifidobacteria and more bacteroides were observed in the overweight subjects (35). Nondigestible oligosaccharides (NDOs) that elicit a prebiotic effect are attracting attention as vehicles for dietary management and the control of obesity and/or metabolic syndrome. Although evidence comes mostly from animal models, NDOs have been shown to regulate food intake and weight gain, dyslipidemia, insulin resistance, hypertension, and liver steatosis (14). Animal models and in vitro experiments have shown that specific immunomodulatory effects are induced by prebiotics (36,37). This immunomodulation is usually thought to be related to the microbiota. However, recent evidence suggests that, depending on the chemical structure of the tested prebiotic, a direct interaction can take place between the prebiotic and the host through binding of the prebiotic to specific receptors on cells of the immune system (38,39).

TABLE 3 Plasma lipids, glucose, and insulin in overweight adults during 12-wk B-GOS and placebo interventions<sup>1</sup>

Intervention/ time point	TC	TG	HDL-C	LDL-C	Glucose	Insulin
TC:HDL-C ratio	mmol/L pmol/L					
Placebo Baseline	6.2	6	1.3	1.6	6	0.7
12 wk	6.1	1.8	6	0.9	1.4	6
B-GOS Baseline	6.3	6	1.3	1.6	6	0.8
12 wk	6.1	5.4	6	0.6	67.3	6

1 Values are mean  $\pm$  SD, n = 45. Following Bonferroni adjustment for multiple testing, significance was set at P , 0.005. No differences were observed between the 2 groups of participants at the beginning of each intervention period. The period-intervention interaction, which tested for a carryover effect, was nonsignificant and therefore removed from the model. The gender-intervention interaction (P , 0.0001) and gender main effect (P , 0.005) were significant only in the case of TG at 12 wk. Asterisks indicate different from placebo at the same time point: \*P , 0.0001, \*\*P , 0.005. B-GOS, Bi2muno; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TC, total cholesterol.

FIGURE 2 Changes in the plasma TG concentration and the TC:HDL-C ratio in overweight men and women who received B-GOS and placebo interventions for 12 wk each. Values are mean  $\pm$  SD, n = 45. Data were analyzed in a mixed model taking the period and gender effect and the interaction between intervention and gender into consideration. Following Bonferroni adjustment for multiple testing, significance was set at P , 0.005. \*Different from corresponding female, P , 0.005; \*\*different from corresponding placebo, P , 0.0001. B-GOS, Bi2muno; HDL-C, HDL cholesterol; TC, total cholesterol.

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The present study assessed the effects of a novel transgalactooligosaccharides mixture (B-GOS), compared with a placebo (maltodextrin), on major groups of bacteria found in the feces, immune function, and markers of metabolic syndrome in overweight adults. To the best of our knowledge, this is the first study that has looked at the effect of an NDO on such components in overweight humans. B-GOS, at the daily intake used in this study, has previously been shown to exert a significant and specific prebiotic effect in various human populations such as healthy adults, sufferers of irritable bowel syndrome, and older adults (16,40,41). Upon the ingestion of B-GOS, the selective fermentation of this prebiotic within the human gut results in a significant increase in the number of bifidobacteria in the feces at the expense of less beneficial groups of Gram-negative bacteria such as bacteroides and Desulfovibrio spp.; these phenomena were observed in the present study. In addition, B-GOS (5.5 g/d) was previously shown to exert significant positive effects upon the immune system of older adults, as evidenced by an improvement in phagocytosis and NK cell activity, decreased secretion of proinflammatory cytokines (IL-6, IL-1b, and TNFa), and increased secretion of antiinflammatory IL-10 (16). In the present study, we did not observe any significant changes in the concentrations of measured cytokines, possibly due to the type of diet and amount of food ingested by the volunteers, which could affect the time it takes to modulate this type of the immune response. It seems that in the case of the

majority of biomarkers measured in this study, the effect of the prebiotic was not significant until the end of the 12-wk intervention period. With respect to modulation of the gut microbiota, although the effect of B-GOS was significant after 6 wk, it was relatively small compared with what was previously reported in younger and older adults fed the prebiotic over a shorter time period (16,41). It could therefore be possible that in overweight adults, due to their dietary habits, modulation of the overall gut environment, including the immune response, takes longer than anticipated. However, significantly lower concentrations of fecal calprotectin (marker of intestinal inflammation) and plasma CRP were observed following the administration of B-GOS compared with placebo. Furthermore, an increase in levels of fecal sIgA, primarily involved in mucosal immunity and protection of barrier function against infection, was also observed after the B-GOS intervention in the present study. The data therefore suggest that B-GOS positively influences the immune response in overweight adults at mucosal and systemic levels. The exact mechanisms by which bifidobacteria exert their positive effects in the human gut are not fully understood. However, a number of different modes of action have been demonstrated in studies in rodent models. Administration of bifidobacteria to rodents has been shown to lead to improved barrier function, improved immune response, and a reduction in inflammatory compounds such as intestinal LPS (exposure to which can lead to metabolic endotoxemia) (42–45). Animal studies and human feeding trials have shown that administration of FOS is effective in lowering BMI, waist circumference, total energy intake, and glucose and insulin concentrations (13,46,47). In the present study, with the exception of insulin, we did not find any significant effects on these measurements nor on BP; significantly lower concentrations of insulin were detected upon the administration of B-GOS. The observed differences of these variables after FOS and B-GOS interventions can be attributed to the differences in the chemical structures of the 2 prebiotics, and thus the way they are utilized by the gut microbiota, the metabolites, and/or interactions of which will influence systemic responses.

Prebiotics and probiotics have also been shown to effectively improve lipid profiles. Whereas animal models suggest the efficacy of these functional foods in lowering TC, TG, and LDL-C concentrations and improving HDL-C, results obtained in humans are variable. Some studies have reported improved lipid profiles through administration of either probiotics (48) or inulin (49), whereas others have shown no such effect (50,51). Most studies reporting decreased concentrations of plasma lipids and/or increased HDL-C following either probiotic or prebiotic administration have used hypercholesterolemic subjects. Our previous study with older subjects who had normal TC concentrations showed that B-GOS did not affect TC or HDL-C (16). However, in the present study, compared with placebo, B-GOS significantly reduced TC, TG, and TC:HDL-C ratios while having no effect on LDL-C and HDL-C concentrations in overweight subjects. Therefore, B-GOS administration is effective in lowering plasma lipids in a population where the majority of subjects are hypercholesterolemic. Although the exact mechanisms by which probiotics and prebiotics modify plasma lipids are unknown, a number of modes of action have been proposed, all of which point toward the involvement of a beneficial microbiota in improving plasma lipid profiles. These include enzymatic deconjugation of bile salts by bacteria, cholesterol binding in the small intestine, incorporation of lipids into bacterial cellular membranes during growth, conversion into coprostanol and fecal excretion, and inhibition of cholesterol synthesis in the liver through the production of SCFAs (52). In conclusion, this

study has shown that B-GOS administration to overweight adults with metabolic syndrome leads to a decrease in the number of Gram-negative, less beneficial bacteria in the fecal microbiota and an increase in the number of beneficial, Gram-positive bifidobacteria. This shift in the microbiota may be responsible for the significant positive effects on the immune response that we have observed, as evidenced by improvements in blood and fecal inflammatory markers (i.e., CRP and calprotectin, respectively) and increased secretion of fecal sIgA. Furthermore, there were significant effects on some metabolic syndrome markers, namely insulin, TC, TG, and the TC:HDL-C ratio, following the administration of B-GOS. Therefore, dietary intervention using B-GOS is not only an attractive option for enhancement of both the gastrointestinal and immune systems in overweight individuals, but it is also potentially beneficial in reducing some of the markers of metabolic syndrome independent of other lifestyle changes, which could be of particular importance in ameliorating the disorders associated with metabolic syndrome.

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#### Inflammatory Bowel Disease: A Potential Result from the Collusion between Gut Microbiota and Mucosal Immune System

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**Abstract:** Host health depends on the intestinal homeostasis between the innate/adaptive immune system and the microbiome. Numerous studies suggest that gut microbiota are constantly monitored by the host mucosal immune system, and any slight disturbance in the microbial communities may contribute to intestinal immune disruption and increased susceptibility to inflammatory bowel disease (IBD), a chronic relapsing inflammatory condition of the gastrointestinal tract. Therefore, maintaining intestinal immune homeostasis between microbiota composition and the mucosal immune system is an effective approach to prevent and control IBD. The overall theme of this review is to summarize the research concerning the pathogenesis of IBD, with particular focus on the factors of gut microbiota-mucosal immune interactions in IBD. This is a comprehensive and in-depth report of the crosstalk between gut microbiota and the mucosal immune system

in IBD pathogenesis, which may provide insight into the further evaluation of the therapeutic strategies for IBD.

Keywords: Inflammatory bowel disease; mucosal immune system; gut microbiota

1. Introduction The exact pathogenesis of inflammatory bowel disease (IBD) is still elusive, but it is generally accepted that the inflammation results from a defective mucosal immune response to intestinal flora in genetically susceptible individuals [1]. A common type of IBD is Crohn's disease (CD), in which inflammation is usually transmural and can be found in any area of the gastrointestinal tract. Another major type of IBD, ulcerative colitis (UC), is characterized by a non-transmural inflammation that usually affects the colon and rectum [2]. The highest occurrence of IBD is in developed countries, such as those in North America and Europe, affecting up to 0.5% of the general population [3]. Since urbanization and rapid industrialization in developing countries, traditional lifestyles have changed greatly [4]. The clear relationship between the lifestyle changes associated with industrialization and the incidence of IBD has prompted exploration into the pathogenesis of IBD [2]. Lifestyle changes during urbanization, including improved sanitation, reduced early life microbial exposure, westernized diet, and increased antibiotic use, have been shown to influence the gut microbiota [5]. Furthermore, several lines of evidence support the hypothesis that disturbance of the relationship between the gut microbiota and the mucosal immune system is involved in IBD pathogenesis [6–8].

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The mammalian gut is colonized by a large number of microorganisms, including bacteria, fungi,

viruses, protists, and helminths, which are collectively called the gut microbiota or the microbiome [9–11]. Microbes take part in many physiological host processes, such as the biosynthesis of certain bioactive secondary metabolites.

Furthermore, the microbiota plays an important role in maintaining the normal intestinal epithelial barrier, immune homeostasis, optimal immune responses, and protection against pathogen colonization [12].

Although most of the gut microbiota are mutualistic or commensal, when “dysbiosis” occurs under

certain circumstances, pathogenic bacterial overgrowth can induce certain inflammatory diseases, such as IBD [13]. In this review, we will discuss the collusion between the gut microbiota and the mucosal immune system during the development of IBD.

## 2. Microbiota Dysbiosis as a Potential Trigger for IBD

2.1. Specific Pathogenic Microbes in IBD The gut lumen has a large mucosal interface (300–400 m<sup>2</sup>) that has structures and functions related to immunological recognition of the xenobiotics from the environment [14].

The result of early research suggested that specific pathogenic microbes caused IBD, because many infectious pathogens result in diarrhea and lead to intestinal mucosal inflammation, similar to IBD [15]. *Mycobacterium avium* subspecies *paratuberculosis* is one such pathogen, and it has been widely studied for its potential role in the pathogenesis of CD [16,17]. Although the association of pathogenic microbes with CD seems to be specific, further studies on its regulation in the etiology of CD remain to be defined [18,19]. Another pathogenic microorganism attracting research interest is adherent-invasive *Escherichia*

coli (AIEC). There is growing evidence that AIEC may contribute to the pathogenesis of IBD, especially CD [20]. Compared with healthy subjects, the AIEC richness index in CD patients is significantly increased, and a study has shown that the AIEC protease Vat-AIEC can contribute to intestinal mucosal injury and bacterial colonization [21]. Defensins secreted by Paneth cells play an important role in intestinal mucosal immunity, and intestinal mucosal cell surfaces with high concentrations of defensin also have high AIEC concentrations, suggesting that AIEC might have developed resistance to defensins [22]. A new strain of AIEC, LF82, has been shown to enter and survive in lamina propria macrophages and intestinal epithelial cells (IECs), followed by nuclear factor (NF)- $\kappa$ B signaling activation and TNF $\alpha$  secretion [23]. A recent study by Viladomiu et al. found that interleukin (IL)-17+ CD4+ T cells and ROR $\gamma$ t+ CD4+ T cells were increased in both the colonic and small intestinal lamina propria after AIEC 2A colonization of germ-free C57BL/6 mice [24]. This indicates that AIEC 2A can increase Th17 polarization and effect mucosal immunity. All in all, a large amount of evidence shows that AIEC may contribute to the development of CD, while the signaling pathways involved in intestinal mucosal immunity remain less clear.

**2.2. Profiles of the Intestinal Bacteria and IBD** In recent years, with the development and application of high-throughput sequencing, new techniques (e.g., 16S ribosomal RNA genes sequencing) have provided new approaches for exploring the effect of the gut microbiota in the pathogenesis of IBD [15,25]. Studies have been able to explore the whole bacterial community structure rather than a single or a few bacterial species. An increasing body of evidence suggests that neither a single nor a few pathogenic bacteria, but rather the change in the whole bacterial community structure, may cause IBD [26,27]. Research based on 16S rDNA sequencing has highlighted that only 7–9 of the 55 known bacterial divisions or phyla are detected in human fecal or gut mucosal samples [11]. Bacteroidetes (16%–23%) and Firmicutes (49%–76%) are the most abundant human gut bacteria, and less abundant phyla include Proteobacteria, Fusobacteria, Actinobacteria, and Verrucomicrobia [14,28,29]. Co-evolutionary relationships have been found between the host and symbiotic bacteria (including commensals and mutualists) [30]. Changes in host age, diet, or antibiotic use can cause a shift in symbiotic bacteria. In a healthy human body, after a temporary

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shift, the fecal bacteria have a tendency to return to its typical original structure [31]. The bacterial component of the microbiota provides considerable benefits to the host by generating metabolites, promoting the development of the mucosal immune system, and preventing colonization by pathogenic microorganisms [32]. However, after developing IBD, intestinal micro dysbiosis (imbalance between protective and harmful bacteria) is often found [27]. A widely recognized hypothesis is that intestinal micro dysbiosis can be a trigger for IBD [27]. Intestinal micro dysbiosis has been extensively described in patients with IBD. For example, reduction in diversity, changes in composition (increased or decreased abundance of specific species), and changes in metabolites occur [14,33,34]. Regarding the reduction in diversity, mucosal biopsies from twin pairs (including dizygotic and monozygotic twins) with UC have shown a reduction in gut microbiota diversity in both siblings relative to healthy individuals, indicating a reduction in the diversity of gut microbiota may contribute to IBD [35]. Additionally, studies of bacteria from UC patients

also showed a lower fecal bacterial diversity than healthy individuals [36]. Moreover, in the first two years of life, lower diversity of bacteria in the gut is related to a reduction in T helper 1 (TH 1) responses, which may contribute to the development of IBD in adulthood [37]. Regarding the changes in composition, many studies have shown that the gut microbiota in IBD patient exhibits increased Proteobacteria and reduced Firmicutes [38–40]. Moreover, decreased abundance of Clostridium cluster IV (the Clostridium leptum group), especially *Faecalibacterium prausnitzii*, has been reported [40,41]. Regarding the changes in metabolites, short-chain fatty acids (SCFA) formed by gut microbiota after the digestion of various dietary fibers can be absorbed and utilized by IECs [34]. A total of 95% of SCFAs may be allocated to their rapid absorption, with only about 5% being passed out of the body in the feces. Acetate, propionate, and butyrate are the main components in SCFAs, of which acetate can be produced through the Wood–Ljungdahl pathway by *Blautia hydrogenotrophica*, and propionate is generated by Bacteroidetes and Firmicutes through the succinate and lactate pathway, and the remaining butyrate being produced by several Firmicutes through Acetyl-CoA [42]. It has long been known that European children, who are more susceptible to IBD, have worse fiber digestive capability and lower SCFA levels than African children [43]. On the other hand, other evidence shows that SCFAs involve in regulating immunity and controlling inflammation, suggesting the role of SCFAs in maintaining intestinal homeostasis [34]. Nevertheless, studies on mucosal biopsies from IBD patients have revealed an increase in members of the Enterobacteriaceae family and a decrease in members of the Clostridiales order [43]. In colonic specimens, bacteria (including bacteria of the gamma subdivision of Proteobacteria) were found to invade the mucosa in 83% and 25% of UC and CD patients, respectively, compared with 0% of the controls without IBD [44,45]. In vitro experiments have indicated that several strains of bacteria from CD or UC patients, including *E. coli*, *Enterococcus faecalis*, and *Fusobacterium varium*, can erode IECs [46–48]. However, emerging technologies (e.g., DNA sequencing technologies and computational tools) have also drawn researchers' attention to other gut microbes, such as fungi and viruses [49].

2.3. Fungal Microbiota and IBD Whole-genome sequencing analysis indicates that >99% of the gut microbiota is bacteria, while fungi only account for 0.1% [29].

However, fungi have been suspected to be involved in the pathogenesis of IBD for a long time. Many years earlier, researchers regarded anti-Saccharomyces cerevisiae antibodies (ASCA) as a kind of serological biomarker for CD, indicating an excessive immune response to fungi in CD patients [50,51]. Furthermore, ASCA can be detected in 50%–60% of CD patients compared with only 8%–20% of healthy subjects [52]. Recent studies show that alterations in the fungal community composition and structure also exist between IBD patients and healthy subjects. IBD patients have a decreased Ascomycota/Basidiomycota ratio compared with healthy individuals, which involves an increased abundance of *Candida albicans* and a decreased abundance of *Saccharomyces cerevisiae* in IBD patients [53].

Fungal diversity is also dramatically reduced in IBD [53]. Moreover, in a mouse model of colitis, *C. albicans* aggravated intestinal inflammation while *S. cerevisiae* decreased inflammation [54,55].

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Treatment of mice with an antifungal agent increased susceptibility to acute and chronic colitis [56]. Furthermore, the fungal community in the mammalian gut can interact with the immune system via the innate immune receptor Dectin-1 and the Card9-Syk signaling axis, maintaining intestinal homeostasis [53,54]. These findings provide objective evidence that the fungal “mycobiota” regulate the immune system and impact the incidence of IBD.

**2.4. Enteric Virome and IBD** The intestine contains a large and complex viral community, which is known as “the enteric virome” [57]. The development of metagenomics has helped researchers to reveal the diverse composition of the enteric virome, which contains eukaryotic viruses (e.g., herpesviruses, adenoviruses, and uncharacterized eukaryotic viruses) and prokaryotic viruses (e.g., Microviridae and Caudovirales) [58,59]. However, so far, little is known about the role of the enteric virome in IBD. Recent animal studies have indicated that the enteric virome is involved in the pathogenesis of IBD. A eukaryotic virus, murine norovirus (MNV), disrupted gut homeostasis in IBD-susceptible mice (IL10<sup>-/-</sup> and Atg16L<sup>-/-</sup> mice) and induced serious colitis [60,61]. However, a model of bacteriophage adherence to mucus indicated that there is a symbiotic relationship between bacteriophages and the intestinal mucosa, that is, the mucus provides a habitat for bacteriophages, which provide defense against other microbes [62].

Moreover, a study of MNV infection of germ-free or antibiotic-treated mice found that MNV contributed to restoring the normal intestinal morphology and maintaining their innate immune functions [63]. Furthermore, research on the enteric virome in healthy subjects suggests that bacteriophages comprise much of the virome, and the species are relatively stable [64,65]. Microviridae and Caudovirales, which latently infect their bacterial hosts and generate offspring, representing remain dominant bacteriophage species [66,67]. However, alterations in bacteriophage species composition, also known as dysbiosis of the enteric virome (that is, increased levels of bacteriophages, particularly Caudovirales) have been found in IBD patients. Most interestingly, there is a predator–prey relationship between bacteriophages and their bacterial hosts, which is called a “transkingdom interaction,” and which may contribute to disease pathogenesis [11,57]. In summary, enteric virome affects the mucosal immunity at least in some respects, but its relationship to intestinal homeostasis remains to be investigated.

**2.5. Protozoans and IBD** Although it is clear that the dysbiosis of bacteria, fungi, and viruses can impact intestinal homeostasis, the potential homeostasis-maintaining role of other microbial kingdoms, such as Protista, has seldom been studied. It is generally known that intestinal pathogenic protozoans, which are unicellular eukaryotes including *Cryptosporidium* spp., *Giardia* spp., *Entamoeba histolytica*, *Encyrtophyton cuniculi*, and *Toxoplasma gondii*, can cause diseases in mice and humans [68–72]. Furthermore, it was traditionally believed that any protozoan in human intestines was a parasite that could cause pathogenicity in the host body [73]. However, interestingly, emerging evidence suggests that some common protozoa inhabiting the human intestines are beneficial rather than harmful [74]. A growing body of research demonstrates that intestinal protozoans, such as *Blastocystis* and *Dientamoeba fragilis*, are also found at high levels in healthy individuals [75]. Many other symbiotic protozoans (e.g., *Entamoeba dispar* and *Pentatrichomonas*) are also present in the intestines [76]. There has been little research on the effects of intestinal protozoans on the development of IBD, especially regarding their effects on intestinal mucosal immunity. *Trichomonas musculus* (T.mu), a

commensal intestinal protozoan of rodents, colonizes the bowel lumen and leads to inflammasome activation in epithelial cells and IL-18 and IL-1 $\beta$  release. T.mu-driven IL-18 can protect the intestinal mucosa against bacterial invasion but also promote the development of chronic colitis in mice [77]. Another *Trichomonas* species, *Trichomonas muris*, can dramatically increase the abundance of intestinal tuft cells (critical sentinels in the intestinal epithelium) and then affect type 2 innate lymphoid cells (ILC2s) via Trpm5 and the expression of cytokines, such as IL-25 and IL-13 [78]. Notably, a new

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hypothesis is that intestinal pathogenic protozoans need certain stimulatory factors (e.g., transkingdom interactions with certain intestinal bacteria) to activate their pathogenicity [79].

However, the protozoa, whether pathogen or commensal, remains perplexing.

There is no clear evidence that certain protozoans are useful to support intestinal health. The potential benefits of intestinal protozoans may be derived from increased intestinal biodiversity or their ability to regulate the host intestinal mucosal immunity.

2.6. Helminths and IBD The “IBD hygiene hypothesis” proposed that bringing up children in extremely sanitary environments (e.g., with lower exposure to helminths) adversely affects the construction of the innate

immune system, which contributes to susceptibility to IBD in later life [80].

Many recent clinical studies

have demonstrated that various helminths (e.g., *Trichuris trichiura*, *Trichuris suis*, and *Necator americanus*)

can alleviate IBD symptoms, and their absence has been associated with the development of IBD [5, 81]. The most likely underlying mechanism is that helminths can alter immune responses

(depress or decrease the release of inflammatory factors) in their hosts by releasing various excretory–secretory (ES) products [82]. Research on the effects of helminth

infection utilizing an IBD-susceptible mouse model (nucleotide-binding oligomerization domain-containing protein 2 [Nod2]-knockout mice) has shown that parasitic *Trichuris muris* can ameliorate abnormal intestinal barriers (increasing the quality of goblet cells)

and alter the balance of commensal and pathogenic bacteria [83,84]. Moreover, various

helminths, such as *Echinococcus granulosus*, *Trichinella spiralis*, *Heligmosomoides polygyrus*,

and *Ancylostoma caninum*, have been shown to protect against colitis in animal models [81]. Additionally, Sj16, a secreted protein of *Schistosoma japonicum*, has

immunoregulatory protective effects on dextran sulfatesodium (DSS)-

induced colitis by inhibiting the peroxisome-proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ )

signaling pathway, increasing Treg percentages and up-regulating anti-inflammatory

factors production [81]. As mentioned above, intestinal helminth infection may protect

against IBD through the regulation of multiple immune responses.

### 3. Mucosal Immune System and Intestinal Homeostasis

3.1. Composition of the Mucosal Immune System The intestinal mucosal immune system comprises three barriers against harmful factors and maintains intestinal homeostasis [85].

The mucus layer covering the epithelial surfaces of the intestinal lumen is the first barrier.

This barrier is composed of a complex polymeric network of highly glycosylated mucins

(MUC proteins), which keeps microorganisms away from the IECs [86]. The second barrier

is the single layer of IECs organized in intestinal structures, which is composed of multiple cell types, including goblet, enteroendocrine, tuft, columnar epithelial, and M cells. The

third barrier is the numerous immune cells residing in the gut or scattered throughout the gut epithelium and lamina propria, including the mesenteric lymph nodes and Peyer's patches [85]. All three barriers are important for preventing commensal microorganisms' access to the systemic circulation and maintaining intestinal homeostasis, and any damage or functional abnormality of these barriers may cause CD and UC [87,88].

**3.2. Mucus Layer** The mucus of the large intestine is largely produced and secreted by goblet cells; this mucus layer forms a complex network to produce a physical and biochemical barrier in the colon [89]. This barrier includes two layers, the inner and outer mucus layer. In a healthy gut, the inner mucus layer is impregnable to any commensal microorganisms. However, the outer mucus layer is more exposed to the intestinal lumen and provides a habitat for commensal microorganisms [90]. Mucus mainly comprises mucin glycoproteins, but it also acts as a medium for retaining other proteins, such as antimicrobial peptides (AMPs) and secretory immunoglobulin A (SIgA). Mucin 2 (MUC2) is a kind of gel-forming mucin that is most highly expressed in the colon, forming a stable well-organized

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structure that is almost completely free from bacteria [91]. SIgA, which is secreted across the IECs by plasma cells, is the main antibody of mucosal immunity and binds to pathogens to prevent their direct interaction with the host [92]. In a healthy human, the colonic epithelium is covered by the mucus, but in IBD patients, the percentage of the epithelium covered by mucus is significantly decreased, and the mucus is thin and damaged [93].

An integrated mucus layer ensures that there is no direct contact between pathogens and IECs [93]. In contrast, in several genetic and chemically induced mouse models of colitis, pathogens are close to or even invade the IECs [94]. Moreover, tests on colonic specimens proved that mucosal bacterial invasion is common in IBD patients, while no invasion occurs in healthy controls [49].

**3.3. Single Layer of IECs** The main structure of the intestinal barrier is formed by IECs, which not only create a physical barrier between symbiotic and pathogenic microbes and the lamina propria, but also play a prominent role in intestinal immunity against pathogenic bacteria and their components (e.g., lipopolysaccharides, LPS) [95]. Tight junction formation, mucus, and AMP secretion are examples of the immune function of IECs.

IECs can be divided into absorptive cells (columnar epithelial cells) and secretory cells (goblet, enteroendocrine, and tuft cells) according to their biological functions [96]. Columnar epithelial cells, which are responsible for absorbing digested nutrients, are the main absorptive enterocytes in the intestinal epithelium [97]. Goblet cells, an indispensable secretory-type IECs, can synthesize and secrete gel-forming mucin, especially MUC2 [96]. Enteroendocrine cells, which represent about 1% of IECs, can release gut hormones to control gut movement and regulate food intake [98]. Tuft cells tend to be found later than other types of IECs, and they account for about 0.4% of IECs. Recently, it has been found that tuft cells act as critical guards in the intestinal mucosal immune system, promoting the recognition of and immunity against intestinal parasites [78,99]. Thus, IECs play a crucial role in maintaining intestinal homeostasis and participating in commensal–host interactions.

### 3.4. Intestinal Immune Cells

There are many kinds and a large number of immune cells in the intestines, which play key roles in maintaining intestinal homeostasis.

Changes in their morphology and functions may lead to IBD [100]. Recently, a growing number of studies have begun to focus on the relationships between immune cells and the intestinal microbiota, and research has revealed that the maturity of some immune cells is dependent on specific microbiota (e.g., some Bacteroidetes and Firmicutes species) [100]. Currently, the most studied intestinal immune cells are dendritic cells (DCs), macrophages, adaptive immune cells, and innate lymphoid cells (ILCs). Macrophages and DCs are the main antigen-presenting cells found

under the IECs, which can identify both innocuous antigens and potential pathogens, ensuring that the host responds appropriately to the intestinal microbiota [101,102].

Adaptive immune cells are a type of immune cell that only participate in the adaptive immune response. They can undergo a complex process involving development, differentiation, maturation, and secretion after being stimulated by specific antigens.

Key adaptive immune cells involved in the pathogenesis of IBD are T cells (including the T helper cells Th1, Th2, and Th17, and regulatory T [Treg] cells) [103]. ILCs are also an important class of immune cells that act as guards in the host protective immune system and also participate in immune-mediated diseases.

It has been demonstrated that ILCs respond rapidly to intestinal ecosystem factors, such as luminal bacteria, metabolic signals, and cytokines [104]. It has also been demonstrated that some subsets are involved in the pathogenesis of IBD (NCR- ILC3, ILC1). Additionally, some probably have protective functions (NCR+ ILC3) while others remain controversial (ILC2) [104,105]. Thus, the numerous intestinal immune cells have immune functions in the mucosal immune system (Figure 1).

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Figure 1. Cross-kingdom biological transmission and communication maintain intestinal homeostasis. Intestinal homeostasis is maintained by three immunological barriers: mucus layer (first barrier), epithelium layer (second barrier), and immune cell layer (third barrier). The mucus layer contains multiple immune mediators such as antimicrobial peptides (AMPs) and secretory immunoglobulin A (SIgA), which limit direct contact between the millions of microorganisms (including bacteria, fungi, viruses, and protists) and the intestinal epithelial cells (IECs).

However, microorganisms are responsible for the degradation and digestion of dietary fiber to produce high-energy materials (e.g., short-chain fatty acids [SCFAs]) for the IECs. The IEC layer, which contains multiple pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nod-like receptors (NLRs), is the second immunological barrier. It rapidly detects and responds to bacteria that invade the intestinal tissue. Finally, the immune cell layer promotes the monitoring and clearance function of the IECs to limit the access of enteric microbes, thus ensuring that “unlucky” invaders are killed rapidly while also promoting intestinal homeostasis. 4. Orchestrated Balance between Mucosal Immune System and Gut Microbiota

4.1. Interaction between Treg/Th17 Axis and Gut Microbiota Tregs, Th1, Th2, and Th17 are all derived from the differentiation of naïve CD4+ T lymphocytes, which can be promoted by ILCs, DCs, and macrophages [106]. As one of the most studied CD4+ T helper cell subsets, Th17 cells are characterized by IL-17 production and secretion, which promotes

intestinal inflammation [107]. Th17 cells are crucial for protecting the intestinal mucosal barrier from pathogens, comprising bacteria, fungi, and viruses [108]. However, in IBD patients, the majority of gut Th17 cells are found in ulcerative areas, and advanced mice experiments have also shown that abnormally elevated levels of Th17 cells (induced by specific bacteria) can exacerbate colitis [24]. Tregs are a subset of CD4<sup>+</sup> T cells, and they are defined by the expression of CD25 and Foxp3 [109]. Tregs play a crucial role in the negative control of the immune system by producing IL-10 and transforming growth factor (TGF)- $\beta$ , maintaining immunotolerance and immune homeostasis. Studies have shown that Treg defects and functional abnormalities are involved in the pathogenesis of various diseases, including IBD [110,111]. Therefore, the Treg/Th17 axis maintains the intestinal mucosal immune homeostasis and determines the incidence and severity of IBD. Correcting the imbalance of the Treg/Th17 axis may contribute to the alleviation of inflammation. Since the establishment and application of germ-free mice, the relationship between the gut microbiota and the Treg/Th17 axis has been widely studied. Early studies showed that germ-free mice had fewer CD4<sup>+</sup>CD25<sup>+</sup> T cells in mesenteric lymph nodes, suggesting that the gut microbiota favors the development of Treg cells [112]. Moreover, colonization with different types of microbiota or a single bacterial strain can trigger different immune responses and establish diverse gut immune landscapes [113]. In vitro co-culture experiments involving *Clostridium* and colon epithelial cells indicated that *Clostridium* induced TGF- $\beta$  production, which promoted CD4<sup>+</sup> T cell differentiation into Tregs [114]. A major species of the order Clostridia, *Faecalibacterium prausnitzii*, is one of the most abundant anaerobic intestinal bacteria. Research has confirmed that it promotes butyrate production and blocks the IL-6/Stat3/IL-17 pathway, thus reducing CD4<sup>+</sup> T cell differentiation into Th17 cells and promoting Treg cells [115]. *Helicobacter pylori*, a pathogenetic Gram-negative bacterium, can cause gastric ulcers; however, research has indicated that it can ameliorate DSS-induced chronic colitis in mice, which may be associated with Th17 downregulation and Treg upregulation [116]. In brief, all these observations in mouse models supported the hypothesis that changes in the gut microbiota composition alter the balance of the Treg/Th17 axis, contributing to the aggravation or alleviation of IBD [117].

Subsequently, microbiota transfer from healthy or IBD donor to germ-free mice verified the correctness of this hypothesis [118].

As a crucial transcription factor for maintaining the balance of the Treg/Th17 axis, c-Maf can regulate the differentiation and function of intestinal Treg cells. Research has shown that, in c-Maf-deficient mice, the intestinal microbiota was severely disturbed, and when transferred to germ-free mice, the microbiota induced severe intestinal Th17 responses and aggravated inflammatory reactions [119]. Moreover, the IL-17 receptor (IL-17R), which is a key IL-17 signaling pathway receptor responsible for driving Th17 cell development, is essential for regulating the effects of the mucosal immune system against intestinal pathogen infections and controlling gut microbiota dysbiosis [120]. As an indispensable subset of Treg cells, pTreg cells enriched in the intestines have a profound impact on intestinal microbial communities, and pTreg cell deficiency in mice induced pervasive changes in gut microbial metabolite profiles and the intestinal epithelium [121]. In brief, there is a sophisticated crosstalk between the Treg/Th17 axis and the gut microbiota. A Treg/Th17 axis imbalance can cause microbiota dysbiosis, and microbiota

dysbiosis can also lead to the imbalance of the Treg/Th17 axis.

Moreover, disorders of the Treg/Th17 axis or the gut microbiota can lead to or aggravate IBD (Figure 2).

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Figure 2. Alteration of the intestinal homeostatic balance promotes the pathogenesis of inflammatory bowel disease (IBD). During homeostasis in healthy intestines, gut microbes induce an immune tolerance phenotype. In contrast, the key features of homeostasis imbalance are microbiota dysbiosis and immunological dysregulation. Microbiota dysbiosis involves the excessive reproduction of potentially pathogenic microorganisms, which can erode the intestinal mucosa and increase intestinal permeability, thus promoting the overactivation of the adaptive and innate immune system and driving chronic inflammation. Moreover, microbiota dysbiosis can induce imbalance of the Treg/Th17 axis, leading to further inflammatory responses in the intestinal tissue. However, some gut

microbiota (e.g., *Clostridium*, *Faecalibacterium prausnitzii*, and *Helicobacter pylori*) favors the development of Treg cells (Figure 2). Alteration of the intestinal homeostatic balance promotes the pathogenesis of inflammatory bowel disease (IBD). During homeostasis in healthy intestines, gut microbes induce an immune tolerance phenotype. In contrast, the key features of homeostasis imbalance are microbiota dysbiosis and immunological dysregulation. Microbiota dysbiosis involves the excessive reproduction of potentially pathogenic microorganisms, which can erode the intestinal mucosa and increase intestinal permeability, thus promoting the overactivation of the adaptive and innate immune system and driving chronic inflammation. Moreover, microbiota dysbiosis can induce imbalance of the Treg/Th17 axis, leading to further inflammatory responses in the intestinal tissue.

However, some gut microbiota (e.g., *Clostridium*, *Faecalibacterium prausnitzii*, and *Helicobacter pylori*) favors the development of Treg cells to promote the anti-inflammatory effect.

In brief, any side or both abnormal in gut microbiota or Treg/Th17 axis may cause intestinal homeostatic imbalance. Ultimately, disorders of the intestinal homeostasis can lead to or aggravate IBD.

4.2. Communication between Pattern Recognition Receptors (PRRs) and Gut Microbiota PRRs are widely expressed in various cells of the intestinal mucosal immune system, including IECs, DCs, macrophages, adaptive immune cells, and ILCs. They are responsible for recognizing microorganisms' different molecular patterns, thus preventing pathogen invasion and maintaining intestinal homeostasis [121–125].

A growing number of studies are finding that PRRs play a key role in

both avoiding direct contact between gut microbiota and IECs and influencing the structure of intestinal communities [126]. At present, known PRRs include Toll-like receptors (TLRs), NOD domain-like receptors (NLRs), melanoma differentiation-associated gene 5 (MDA5), laboratory of genetics and physiology gene 2 (LGP2), and retinoid acid-inducible gene-1 (RIG-I). However, among all the PRRs, TLRs and NLRs are the classical PRRs, and they have been widely studied and explored [127,128] (Figure 3).

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Figure 3. Diagram showing the activation pathways of toll-like receptors (TLRs) and NOD-like receptors (NLRs) in the intestinal epithelial cells. Intestinal epithelial cells (IECs) express multiple pattern recognition receptors (PRRs) including TLRs and NLRs, which can recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). TLRs are present in cell membranes and endosomes. When TLRs sense PAMPs or DAMPs, they can recruit signaling adaptors (myeloid differentiation factor 88, MyD88) and then initiate a signaling cascade in MyD88 dependent mechanism, eventually causing the transcriptional activation of nuclear factor kappa-B (NF- $\kappa$ B). TLRs can also be activated in the MyD88 independent mechanism that involves TIR-domain-containing adaptor protein inducing interferon- $\beta$  (TRIF). Canonical activation of NLR family pyrin domain-containing proteins (NLRPs) requires two signals. Signal 1 is activated by PAMPs or DAMPs through TLRs for the upregulation of pro-IL-18 and pro-IL-1 $\beta$ . Signal 2 involves the sensitization of NLRs and the assemble of inflammasome, which further induces the activation of caspase-1 to cleave pro-IL-18, pro-IL-1 $\beta$ , and Gasdermin D. Eventually, IL-18, IL-1 $\beta$ , and Gasdermin D N-terminal domain induce cell pyroptosis. TRAF: TNF receptor-associated factors; IRAK: IL-1R-associated kinases; IKK: inhibitor of NF- $\kappa$ B kinase; I $\kappa$ B $\alpha$ : inhibitor of NF- $\kappa$ B $\alpha$ ; IRF: interferon regulatory factors; TBK1: TANK-binding kinase 1; PSA: Polysaccharide A; ssRNA: single-stranded RNA; dsRNA: double-strand genomic RNA; CpG DNA: CpG-rich hypomethylated DNA motifs in microbial genome; GNBPs: Gram-negative bacterial peptidoglycan; MDP: muramyl dipeptide. Figure 3.

Diagram showing the activation pathway of toll-like receptors (TLRs) and NOD-like receptors (NLRs) in the intestinal epithelial cells. Intestinal epithelial cells (IECs) express multiple pattern recognition receptors (PRRs) including TLRs and NLRs, which can recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). TLRs are present in cell membranes and endosomes. When TLRs sense PAMPs or DAMPs, they can recruit signaling adaptors (myeloid differentiation factor 88, MyD88) and then initiate a signaling cascade in MyD88 dependent mechanism, eventually causing the transcriptional activation of nuclear factor kappa-B (NF- $\kappa$ B). TLRs can also be activated in the MyD88 independent mechanism that involves TIR-domain-containing adaptor protein inducing interferon- $\beta$  (TRIF). Canonical activation of NLR family pyrin domain-containing proteins (NLRPs) requires two signals.

Signal 1 is activated by PAMPs or DAMPs through TLRs for the upregulation of pro-IL-18 and pro-IL-1 $\beta$ . Signal 2 involves the sensitization of NLRs and the assemble of inflammasome, which further induces the activation of caspase-1 to cleave pro-IL-18, pro-IL-1 $\beta$ , and Gasdermin D. Eventually, IL-18, IL-1 $\beta$ , and Gasdermin D N-terminal domain induce cell pyroptosis. TRAF: TNF receptor-associated factors; IRAK: IL-1R-associated kinases; IKK: inhibitor of NF- $\kappa$ B kinase; I $\kappa$ B $\alpha$ : inhibitor of NF- $\kappa$ B $\alpha$ ; IRF: interferon-regulatory factors; TBK1: TANK-binding kinase 1; PSA: Polysaccharide A; ssRNA: single-stranded RNA; dsRNA: double-strand

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genomic RNA; CpG DNA: CpG-rich hypomethylated DNA motifs in microbial genome; GNBPs: Gram-negative bacterial peptidoglycan; MDP: muramyl dipeptide. 4.3. TLRs and the Gut Microbiota TLRs, the best-characterized transmembrane receptors, with at least 13 types, exist in various intestinal cells including IECs (e.g., Paneth cells and goblet cells) and resident immune cells in the intestinal lamina propria (e.g., macrophages and adaptive immune cells) [129].

Studies have confirmed that TLR1 to TLR9 all exist in IECs [130], but the exact mechanisms of TLRs underlying the regulation of intestinal homeostasis have yet to be fully illuminated. Bacterial cell walls lipoproteins, bacterial peptidoglycan, and fungal zymosan are recognized by TLR1, TLR2, and TLR6, respectively [131–133]. TLR4 is responsible for recognizing LPS produced by Gram-negative bacteria. TLR5 can recognize flagellin proteins, which are granular proteins constituting bacterial flagellum fiber [129]. Myeloid differentiation primary response gene 88 (MyD88) was identified as the TLR signaling pathway adaptor protein responsible for transmitting the TLR signal to downstream kinases [134].

MyD88 signaling regulates the production of certain AMPs in specialized IECs, maintaining the barrier functions of the intestinal epithelium [135]. MyD88-deficient mice cannot block pathogenic bacterial invasion into the intestinal epithelium [136]. TLR2 can recognize anti-inflammatory *Bacteroides fragilis* polysaccharide A (PSA) and initiate signaling to regulate the Treg/Th17 axis, thereby promoting immunologic tolerance [137]. PSA is only found in the human microbiome, and it activates an anti-inflammatory immune response that alleviates inflammatory disease [138]. However, the exact mechanism of TLR2 involvement in the development of IBD has not been fully elucidated because of paradoxical results in TLR2<sup>-/-</sup> mice [139,140]. TLR5<sup>-/-</sup> mice tend to develop colitis or systemic inflammation, and further research has shown that the mechanism is related closely to *E. coli* due to the altered gut microbiota composition in these mice [141]. Moreover, research has shown that genetic variants of TLR4 in the population lead to susceptibility to IBD [142]. However, TLR4 is highly expressed in colon segments where pathogenic bacterial invasion and infection are exacerbated in DSS-induced colitis [143]. As such, many studies have confirmed that TLRs communicate with the gut microbiota so as to mediate inflammatory immune responses and maintain intestinal epithelial homeostasis.

4.4. NLRs and the Gut Microbiota NLRs expressed in the cytosol are essential for preventing the invasion of pathogenic bacteria. NLRs exist in various intestinal cells, including IECs and resident immune cells in the intestinal lamina propria [144,145]. At least 23 NLR proteins have been identified, but the mechanisms and biological functions of only a minority have been extensively studied [146,147]. NLRs are novel receptors that maintain intestinal epithelial homeostasis via communication and interaction with the gut microbiota. Remarkably, many NLR genes have been characterized as IBD susceptibility genes, as supported by several studies [148–150]. Some NLRs form multimolecular protein complexes, known as inflammasomes, with pro-caspase-1 and apoptosis-associated speck-like protein containing a CARD (ASC). These inflammasomes are assembled upon stimulation by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). Furthermore, activated NLR inflammasomes can trigger caspase-1 activation and induce the production of mature IL-1 $\beta$ /IL-18, thereby provoking an immune response [151,152]. NOD1 can detect a unique  $\gamma$ -D-glutamyl-meso-diaminopimelic acid motif found predominantly in Gram-negative bacterial peptidoglycan, so as to initiate an inflammatory response [153]. NOD2 can recognize muramyl dipeptide (MDP) contained in peptidoglycan, which is found in Gram-positive and Gram-negative bacteria [154].

Exposure to MDP triggers a series of acute inflammatory signaling effects, inducing the production and secretion of inflammatory cytokines [155]. The most investigated NLR, NLR

family pyrin domain-containing protein 3 (NLRP3), can be activated by various exogenous and

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endogenous ligands or stimuli, such as reactive oxygen species, ATP, bacteria, viruses, and fungi [156]. However, how NLRP3 maintains intestinal homeostasis remains controversial. Some studies show that NLRP3<sup>-/-</sup> mice are prone to colitis, while other studies indicate that inflammatory reactions are reduced in these mice [157–159]. NLR family CARD domain-containing protein 4 (NLRC4) ligands include flagellin of bacteria such as *Salmonella* and *PrpJ* and *CprI* (subunits of bacterial type III secretion systems) [160–162]. NLRC4 protects the intestinal mucosal barrier by restricting intestinal pathogens such as *Citrobacter rodentium* and *Salmonella* [160,163]. The NLRP6 ligands remain unknown, but evidence has established a relationship between NLRP6 and the gut microbiota. Microbial genome sequencing has indicated that the gut microbiota is changed in NLRP6-deficient mice, with the levels of dominant bacteria (Firmicutes, Bacteroidetes, and Proteobacteria) being significantly altered [164,165]. Most interestingly, excessive NLRP12 activation inhibits NF- $\kappa$ B signal transduction. NLRP12<sup>-/-</sup> mice also have the same characteristics, reflecting susceptibility to colitis and microbiome dysbiosis, which indicates the key role of NLRP12 in maintaining intestinal homeostasis [166].

5. Discussion and Conclusions When intestinal homeostasis is maintained, the intestinal mucosal immune system can effectively

resist pathogen invasion and inhibit excessive pathogen reproduction, and simultaneously, commensal intestinal bacteria maintain intestinal immune tolerance [167]. However, immune system–microbiota interactions act as a double-edged sword, with the microbiota being beneficial to the host in normal conditions, but also potentially causing adverse effects in the host that contribute to inflammation [168].

The gut microbiota is constantly monitored by the mucosal immune system, and any slight disturbance in the gut microbiota may contribute to intestinal immune disruption and increased susceptibility to IBD [169]. The intestinal mucosal immune system contains various signal transduction pathways that involve PRR signaling and adaptive T cell responses. PRRs are the first sensors of microorganisms (including pathogens, commensal bacteria, and conditional pathogens), and they act as part of the host defense system. However, it is not clear whether all microorganisms (such as bacteria, fungi, or viruses) are equally sensed by PRRs or whether there are more specific recognition and defense mechanisms for maintaining intestinal homeostasis. Moreover, although plenty of studies have shown the effects of pathogens and commensal bacteria on intestinal immune function, the influences of conditional pathogens on intestinal mucosal immune homeostasis are rarely reported. Nevertheless, a certain co-evolutionary relationship has been found between conditional pathogens and hosts, and conditional pathogens may activate the innate intestinal immune system, thus causing intestinal inflammation [106,170,171]. Interestingly, microorganism exposure in early life is crucial for the construction of the host immune system, and it helps the host to build early innate immune responses and regulate the development of autoimmune and inflammatory diseases such as IBD [5,167,172]. During the first few years of life, intestinal microorganisms can directly or indirectly affect the maturation of the intestinal mucosal immune system [173]. Moreover, *Clostridia* colonization of the neonatal

intestinal tract contributes to the prevention of enteric pathogen growth [174].

Furthermore, by constantly monitoring pregnant mice and their offspring, a recent study showed that maternal microbial exposure

during pregnancy shapes the intestinal immune system of the offspring, including the innate lymphoid and mononuclear cell populations [175].

The intestinal mucosal immunity (including the IEC functions, IgA production, and differentiation of T-cell subsets) of germ-free mice is very different from that of conventionally raised mice, and germ-free mice are more sensitive to DSS exposure [176–179]. Meanwhile, there is a potential link between PRR signaling deficiency (e.g., related to NOD2, MyD88, and TLR5) and the microbiota composition, and defects in certain PRRs may contribute to IBD susceptibility [136,141,180]. Remarkably, mutations of NOD2 loci in IBD patients are significantly correlated with compositional changes in the intestinal-associated microbiota, including increased *Escherichia* and decreased *Faecalibacterium* [181].

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In summary, microbiota dysbiosis may affect the intestinal mucosal immune system and, in turn, immune system dysfunction may cause gut microbiota disorders. The mutual interaction between the intestinal immune system and the gut microbiota may contribute to the pathogenesis of IBD. However, evidence from research on this interactive relationship is still very limited, lacking the construction and comprehension of co-regulation network between signaling pathways and gut microbiota or its metabolites profiles. Adopting multidisciplinary and multi-domain technologies, combining with genomics, proteomics, metabolomics, rapidly maturing computer artificial intelligence and bioinformatics technology will be critical to further illuminate the perplexing mechanisms of gut microbiota-mucosal immune interactions in IBD. Accurately understanding and clarifying the complicated connections between gut microbiota and mucosal immune system, will help researchers to develop novel and effective therapies, and eventually cure IBD.

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Abbreviations AIEC adherent-invasive *Escherichia coli* AMPs antimicrobial peptides ASCA anti-*Saccharomyces cerevisiae* antibodies ASC apoptosis-associated speck-like protein containing a CARD CD Crohn's disease DAMPs damage-associated molecular patterns DCs dendritic cells DSS dextran sulfate sodium IBDs inflammatory bowel diseases ILCs innate lymphoid cells IECs intestinal epithelial cells LGP2 laboratory of genetics and physiology gene 2 LPS lipopolysaccharides MDA5 melanoma differentiation-associated gene 5 MUC2 mucin 2 MDP muramyl dipeptide MNV murine norovirus MyD88 myeloid differentiation primary response gene 88 NLRs NOD domain-like receptors NF- $\kappa$ B nuclear factor kappa-B PAMPs pathogen-associated molecular patterns PRRs pattern recognition receptors PSA polysaccharide A Treg regulatory T RIG-I retinoid acid-inducible gene-1 SIgA secretory immunoglobulin A SCFA short-chain fatty acids TLRs toll-like receptors ILC2s type 2 innate lymphoid cells UC ulcerative colitis

## 28. What are the benefits of a Paleo-type Diet?

- a. Avoids anti-inflammatory foods
- b. Includes dairy products for sufficient calcium
- c. Excludes canola and soybean oils
- d. Both A and C
- e. Both A and B

### Paleolithic Diet

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Introduction A Paleolithic diet is essentially the diet that humans ate during the Paleolithic or “Old Stone Age” era. This period, about 2.5 million years ago, is marked by anatomic and physiologic changes that were taking place in the human body as people adapted to climate change, learned to control of fire, and began to use stone tools. Anthropologists hold that the diet of our ancestors heavily influenced their neural expansion, increased brain size, and reduced gastrointestinal tract size.[1][2][3]

Function So, how did this interest in a prehistoric diet start? And what do health care professionals need to know about the ancient diet pattern's resurgence? The last hundred years have seen a boom of industrialization, leading to the growth of a fast-paced economy. Though industrialization is essential for human advancement, it also has given rise to ultra-processed, low cost, readily available foods to sustain a growing population. A consequence of consuming these foods is a doubling or tripling in the rate of chronic diseases like obesity, diabetes, hypertension, and heart disease. Scientists and laypeople alike have started looking at solutions for these epidemics; alternatives focus not just on medications but the adoption of significant dietary and lifestyle changes. This quest for the “ideal” diet for health and longevity has brought to light several ancient cuisines, some have been thoroughly studied, like the “Mediterranean Diet.” The concept of Paleolithic diet started in the 1970s, and its popularity soared after the publishing of the book *The Paleo Diet: Lose Weight and Get Healthy by Eating the Foods You Were Designed to Eat* by Loren Cordain in 2002. Since then, the public has shown a tremendous interest in this diet, also called the “cave man diet” or “Stone Age diet.” Many cookbooks have been published claiming to have Paleolithic recipes. The idea behind this diet is that if we revert back to what our prehistoric ancestors ate and reject the modern-day, processed diet, our health outcomes would change significantly. The challenge, however, with this diet is that is conflicting versions of this diet are presented to the public, creating confusion. Thankfully, several breakthrough developments in the field of anthropology in the last few years have helped dieters and practitioners better understand the Paleolithic diet. Perhaps one of the most popular misconceptions is that our ancient ancestors were mainly carnivores when, in fact, they mostly ate a plant-based diet. The diet was very broad and was also influenced by the geographical location of the group and food availability.[4][5][6][7] Scientists and anthropologists have been able to reconstruct this diet based on the evidence gathered from archeological remains and studying the modern nomadic tribes. The prehistoric man is also known as the “hunter and gatherer” as agriculture had not started yet. In the modern world, we still have about a dozen or so tribes still following the same hunter and

gatherer concept. These tribes are located in different parts of the world and in all climatic terrains. The most extensively studied tribe is the Hadza tribe from central Tanzania because the African continent is considered the hallmark of human evolution where the majority of prehistoric fossils are found. Since the Hadza group resides in the tropical forest, their diet mainly consists of plants, fruits, tubers, and game animals. One of the most

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Paleolithic Diet - StatPearls - NCBI Bookshelf

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popular food groups for them is honey. There also are studies available on some Nordic tribes, who sustain mainly on fish and other seafood.[8][9][10] Based on these findings, the Paleolithic diet most likely consisted of the following

Plants - These included tubers, seeds, nuts, wild grown barley that was pounded as flour, legumes, and flowers. Since they had discovered fire and using stone tools, it is believed that they were able to process and cook these foods. Animals - Because they were more

readily available, lean small game animals were the main animals eaten. As per some estimates, animal products contributed to only about 3% of the whole diet. Animals were

not yet domesticated so dairy products were probably not included. Seafood - The diet included shellfish and other smaller fish. It was a major component of a diet in the coastal regions. Insects - A variety of insects and their products, including honey, honeycombs,

were eaten. They were a major fallback food. Recently, the interest in edible insects, called entomophagy, has increased. The United Nations released a list of edible insects as an

alternative to meat products as the insects are said to provide similar nutrition benefits. Clinical Significance It is clear that they did eat a variety of high-quality foods that were rich

in nutrients and fiber. Compared to this diet, the diet we eat today provides much less variety and is loaded with artificial sugars and salt. Since it is impossible to mimic the exact

diet that our Stone Age ancestors ate, we can reasonably take some key foods and adapt them to a modern lifestyle. There has not been enough evidence to complete as many

clinical trials on a Paleolithic diet as compared to some of the other diets. Nevertheless, there have been some interesting studies. Whalen KA, et al. have done studies on the Paleolithic

Diet, comparing it to the Mediterranean Diet. In one study of over 2,000 people, participants in each group consumed the list of foods that would fit into each diet pattern.

The results were similar in both the groups, although the consumers of a Paleolithic diet decreased their all-cause mortality, decreased oxidative stress, and also decreased

mortality from cancers, specifically colon cancers. Another study by Blomquist C, et al. involved women who were postmenopausal and also overweight. They found that a

Paleolithic diet decreased lipogenesis promoting factors, improved insulin sensitivity, and reduced circulating triglycerides. The Paleolithic diet also has been studied as a

supplement for therapeutic management in patients with inflammatory bowel disease. An interesting article by Dr. Jacob Eaton and Dr. Lara Lannotti, strong advocates and pioneers

of the Paleolithic Diet, focuses on the mismatch of genomic evolution and the modern day diet. As discussed above, the diet that our ancestors ate has had a major impact on our

genetic evolution. Since today's diet no longer contains the same variety and nutrition, however, there is an increase in chronic diseases caused by both "undernutrition" and

"overnutrition." Multiple other smaller-scale studies confirm similar results. Physicians

across the globe have been trying to incorporate healthy dietary and lifestyle habits into the therapeutic regimen of their patients. A Paleolithic diet is certainly a reasonable option for physicians to choose as it advocates healthy eating.

Enhancing Healthcare Team Outcomes The diet mania continues and recently the paleolithic diet has been hyped up as a cure for most of humankind's health problems. Clinicians should not fall into the trap of 'diet mania' but instead to improve patient outcomes should encourage patients to eat a healthy diet, exercise regularly, discontinue smoking and be physically active. Like all

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diets, the paleolithic diet has its pros and cons, depending on what one reads. This diet first used by our human ancestors may have been good for the digestive tract but most people were dead in their 30s-40s and so, of course, they never developed any serious chronic disorders. Physicians, pharmacists, nurses and dietitians should encourage a healthy diet, similar to a mediterranean diet, which has good clinical evidence to back it up.

Metabolic and physiologic effects from consuming a hunter-gatherer (Paleolithic)-type diet in type 2 diabetes U Masharani<sup>1</sup>, P Sherchan<sup>1</sup>, M Schloetter<sup>2</sup>, S Stratford<sup>1</sup>, A Xiao<sup>1</sup>, A Sebastian<sup>1,2</sup>, M Nolte Kennedy<sup>1</sup> and L Frassetto<sup>1</sup>

**BACKGROUND/OBJECTIVES:** The contemporary American diet figures centrally in the pathogenesis of numerous chronic diseases— 'diseases of civilization'—such as obesity and diabetes. We investigated in type 2 diabetes whether a diet similar to that consumed by our pre-agricultural hunter-gatherer ancestors ('Paleolithic' type diet) confers health benefits.

**SUBJECTS/METHODS:** We performed an outpatient, metabolically controlled diet study in type 2 diabetes patients. We compared the findings in 14 participants consuming a Paleo diet comprising lean meat, fruits, vegetables and nuts, and excluding added salt, and non-Paleolithic-type foods comprising cereal grains, dairy or legumes, with 10 participants on a diet based on recommendations by the American Diabetes Association (ADA) containing moderate salt intake, low-fat dairy, whole grains and legumes. There were three ramp-up diets for 7 days, then 14 days of the test diet. Outcomes included the following: mean arterial blood pressure; 24-h urine electrolytes; hemoglobin A1c and fructosamine levels; insulin resistance by euglycemic hyperinsulinemic clamp and lipid levels. **RESULTS:** Both groups had improvements in metabolic measures, but the Paleo diet group had greater benefits on glucose control and lipid profiles. Also, on the Paleo diet, the most insulin-resistant subjects had a significant improvement in insulin sensitivity ( $r=0.40$ ,  $P=0.02$ ), but no such effect was seen in the most insulin-resistant subjects on the ADA diet ( $r=0.39$ ,  $P=0.3$ ). **CONCLUSIONS:** Even short-term consumption of a Paleolithic-type diet improved glucose control and lipid profiles in people with type 2 diabetes compared with a conventional diet containing moderate salt intake, low-fat dairy, whole grains and legumes. *European Journal of Clinical Nutrition* (2015) 69, 944–948; doi:10.1038/ejcn.2015.39; published online 1 April 2015

**INTRODUCTION** Patients with type 2 diabetes frequently have a number of metabolic abnormalities including insulin resistance, hypertension, dyslipidemia, hyperuricemia and coagulopathy. The underlying mechanisms that lead to the clustering of these abnormalities are not well understood. Genetic factors are implicated, but environmental

factors such as diet are also important.<sup>1–3</sup> Diet can impact the metabolic abnormalities in a number of ways. First, excess caloric intake increases adiposity and insulin resistance. Second, dietary components (for example, fructose, saturated fats, carbohydrates, vitamins and minerals) per se can affect metabolic processes.<sup>4–8</sup> Population and migration studies and analysis of dietary trends indicate that the typical western diet (rich in processed meat, high-fat dairy products and refined grains) is associated with the increased incidence of type 2 diabetes, hypertension and dyslipidemia.<sup>9</sup> Diets metabolically more attuned to human evolution<sup>10</sup>—composed of meats, fish, fruits, vegetables and nuts and excluding processed foods, dairy products and refined grains, the so-called Paleolithic (Paleo-) type diets—could potentially prevent or reverse these disorders.<sup>11</sup> Paleo diets typically are also lower in sodium and very much higher in potassium, antioxidants, micronutrients and fiber and with a much lower diet acid content.<sup>11,12</sup> In a short-term study administering an ad libitum outpatient Paleo diet to healthy volunteers, Osterdahl et al.<sup>13</sup> noted improvements in blood pressure (BP) and weight loss, but no significant improvement in carbohydrate and lipid metabolism.

In another study, Lindeberg and colleagues placed 29 nonhypertensive patients with either glucose intolerance or type 2 diabetes and ischemic heart disease on 12 weeks of either a Paleo diet or a Mediterranean-type diet. They reported lower glucose excursions with oral glucose tolerance tests on the former compared with the latter diet.<sup>14</sup> These two studies did not attempt to control what subjects actually ate nor for improvements related to weight changes. We report here a controlled study of a Paleo diet in type 2 diabetes addressing a number of these confounding factors. We provided all the food with well-defined composition, and we confirmed dietary compliance by composition analyses of several 24-h urine collections. We adjusted caloric intake so as to minimize any weight loss. We compared the Paleo diet with a standard diet based on nutrition recommendations of the American Diabetes Association (ADA diet)<sup>15</sup>

**MATERIALS AND METHODS** Participants Twenty-five patients with type 2 diabetes (aged 50–69 years) were recruited from the San Francisco Bay area. Those who passed a telephone screening by the study investigators were invited for a screening visit. Exclusion criteria included the following: diagnosis of type 1 diabetes; inability to consume the provided diet; pregnancy; hemoglobin  $10\text{g/dl}$ , body mass index (BMI)  $40\text{kg/m}^2$  or on treatments that could affect insulin sensitivity such as thiazolidinediones and glucocorticoids. The study was approved by the UCSF committee on human research, [clinicaltrial.gov](http://clinicaltrial.gov)

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NCT 00548782, and all subjects signed the informed consent. Subjects initially randomized to the ADA diet had the option of participating in the Paleo diet arm after a washout interval of 3 months (five subjects).

Procedures Baseline data (urine collections; electrolytes, lipids profile, hemoglobin A1c (HbA1c), fructosamine, insulin sensitivity, BP) were collected while patients were on their usual diets (days -2 to 0). Then, subjects were randomized either to the Paleo diet or to the ADA diet. There were three ramp-up diets for 7 days, then 14 days of the test diet. All the initial tests were repeated at days 19 to 21. Subjects were also invited to return 1 month after completing the study for repeat testing to determine whether there was a sustained effect of the diet.

Diet. Participants were interviewed by the Clinical Research Center's (CRC's) dietician using a 5-pass, 24-h diet recall on their usual diet. Diet recalls were analyzed using Food Processor SQL (Version 10.0.0) Nutrition Software (ESHA Research Inc., Salem, OR, USA). All study diets were developed using ProNutra Nutrition Software (Version 3.1.0.13, Princeton Multimedia Technologies Corporation, Princeton, NJ, USA). We used a 2-day alternating menu to avoid boredom with the diets. The Paleo diet consisted of meat, fish, poultry, eggs, fruit, vegetables, tree nuts, canola oil, mayonnaise and honey. We excluded dairy products, legumes, cereals, grains, potatoes and products containing potassium chloride. Some foods, such as mayonnaise, carrot juice and domestic meat, were not consumed by hunter gatherers but contain the general nutritional characteristics of pre-agricultural foods. The nutrient composition was verified by composite analysis performed by Covance Laboratories (Covance Labs, Madison, WI, USA; Table 1). The diets were divided into three meals and three snacks, all prepared by the research center kitchen staff. Participants ate some of the meals in the Research Center and the rest of the meals were packed for take-out. Maintenance energy needs were calculated for each individual based on their weight and daily activity levels. To prevent weight loss, body weight was measured every 2 days, and if a deviation of three pounds from the third day's weight in the study occurred calorie intake was adjusted. To allow for adaptation of the subjects' intestinal tract and potassium handling systems to adjust to the markedly higher dietary content of the fiber and potassium in the Paleo diet, a series of ramp diets (with increasing levels of potassium and fiber) were developed. Ramp 1 diet was 1 day, ramp 2 for 3 days and ramp 3 for 3 days and then the Paleo diets were eaten for the remainder of the study. There was no ramp up for the ADA diet.

Blood tests. Fasting blood tests were conducted in triplicate (days -2 to 0 and +19 to 21). Urine collections. Twenty-four-hour urinary sodium and potassium excretion were carried out in triplicate (days -2 to 0, and +19 to 21) and used as a marker for dietary compliance. BP measurements. BPs were measured in the upper arm after 10min of the patients' sitting, at 1min intervals for 3min using an automated BP measuring system (Dinamap, GE Healthcare, Little Chalfont, UK).

Euglycemic hyperinsulinemic clamp. Medications were withheld the morning of the procedure. Insulin was given as an IV bolus ( $0.1\text{U}\times\text{kg body weight}\times\text{desired plasma insulin concentration of }100\text{mU/l}$ ) over 10min followed by a continuous infusion at  $80\text{mU}/\text{min per m}^2$  for 120min. Plasma glucose concentration was maintained at  $5\text{mmol/l}$  by a variable infusion of 20% glucose. Glucose disposal values ( $\text{M}/\text{LBM}/\text{I}$ ) were calculated as  $\text{mg glucose infused per min per kg lean body mass (M/LBM)}$  divided by steady-state insulin levels (in  $\mu\text{U}/\text{ml}\times 100$ ).<sup>16,17</sup>

Body composition. Total body water and extracellular water volumes, surrogate markers for fat and fat-free mass, were determined by bioimpedance spectroscopy (Intermed Inc, Melbourne, VIC, Australia). Subjects were measured supine after resting for ~10min, using

electrodes applied to the hands and feet. Fat-free mass by bioimpedance spectroscopy was used as an estimate of LBM.

**Power calculations.** The primary outcomes for this study were change in insulin sensitivity and improvements in lipid profiles (total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol). The sample size estimate was determined on the basis of our previous study that observed significant improvements in lipid profiles and insulin sensitivity (changes in insulin area under the curve from an oral glucose tolerance test) with nine healthy subjects who were of average fitness by VO<sub>2</sub> max treadmill testing fed a metabolic balance Paleo diet for 2 weeks.<sup>10</sup> This study documented a change in fasting insulin levels with an approximate standard deviation of 15%. For this study, we wanted to detect a minimum difference of 20% between-group fasting insulin levels with a 5% error (that is, Po0.05). The power analysis indicated that each group should have 10 patients per group (2 groups) in order to give a power of 0.8 (that is, an 80% chance to determine a 20% difference between treatments).

**Sample analyses** All of the initial testing was repeated on days 19–21. Repeated test (that is, blood or urine) results for day -2 to 0 and days 19 to 21 were averaged. Blood and urine samples were sent to Quest Diagnostics (San Jose, CA, USA) for sample analysis.

**Statistics** Repeated measures analysis of variance with post hoc paired T-tests or regression analyses were used to evaluate most of the data (Sigmaplot, San Rafael, CA, USA). Unpaired T-tests were used for comparisons between groups. The data in tables are mean±s.d.

**RESULTS** Baseline characteristics Twenty-five subjects enrolled (Table 2) in the study—two subjects decided not to participate after screening; one subject was dropped after being diagnosed with type 1 diabetes; three subjects were lost to follow-up during the trial. Five subjects who initially completed the ADA diet were recruited for the Paleo diet after a washout period of 3 months. Ten subjects completed ADA diet and fourteen subjects completed the Paleo diet. Table 2 summarizes the baseline demographics. Fifteen (62.5%) of the participants identified their

Table 1. Diet composition

2-Day alternating menus kcal Pro, g Fat, g CHO, g SFA, g MFA, g PFA, g Na, mmol K, mmol Ca, mg

Paleo 1	3002	146	99	405	11	53	28	64.7	336.0	977	Paleo 2	3001	131	81	469	13	46	14	72.7															
292.1	887	Average	3001.5	138.5	90	437	12	49.5	21	68.7	314.0	932	ADA 1	3005	123	102	422	24	52	17	4088	6574	2044	ADA 2	2996	182	90	394	19	40	24	4136	6100	1952
Average	3000.5	152.5	96	408	21.5	46	20.5	4112	6337	1998	% calories	Paleo	18.5	27.0	58.2	3.6	14.8	6.3	ADA	20.3	28.8	54.4	6.4	13.8	6.1									

Abbreviations: CHO, carbohydrate; MFA, monounsaturated fatty acid; PFA, polyunsaturated fatty acid; Pro, protein; SFA, saturated fatty acid. Bolded are averages. Paleo diet significantly different from ADA with respect to SFA, Na, K, Ca (Po0.05).

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race as European American/White, three (12.5%) as African American, three (12.5%) as Asian and three (12.5%) as Hispanic. The average BMI was 33.8±8.6 in the ADA group and 32.5±6.0 in the Paleo group. Patients in the ADA group and Paleo group did not statistically

differ on any patient characteristics at baseline (Table 2). Both groups had well-controlled diabetes with HbA1c around 7% and fructosamine levels close to normal.

Medications Four subjects were controlled with diet, fourteen were on metformin alone, five were on a combination of metformin and sulphonylurea and one patient was on sulphonylurea and long acting once daily insulin. The subjects remained on the same doses of diabetes medicines for the duration of the study. Nine subjects were on HMG CoA reductase inhibitors ('statin'); two were on statins and fibrates; five subjects were on an angiotensin converting enzyme inhibitor and one on angiotensin receptor blocker (ARB); one on calcium channel blocker; two were on an ARB and thiazide diuretic; one on  $\beta$ -blocker and ARB; one on ARB,  $\beta$ -blocker and thiazide diuretic; and one on angiotensin converting enzyme inhibitor,  $\beta$ -blocker and loop diuretic. These subjects remained on these drugs at the same doses for the duration of the study. Subjects were asked to stop other diet supplements including fish oils and multivitamins at the time of recruitment. The usual diet was obtained from 24-h dietary recalls by an experienced research dietitian. The ADA and Paleo groups were not different at baseline in terms of sodium and potassium intake.

Changes while on diet Weight changes. The average weight changes were similar in both groups,  $-2.1 \pm 1.9$  vs  $-2.4 \pm 0.7$  kg in the ADA and Paleo diets, respectively,  $P=0.7$ .

Urinary changes. We would expect changes in urinary electrolytes to reflect the diets. The Paleo diet sodium and potassium contents were 69 and 314 mmol/3000 kcal, respectively, and the ADA diet sodium and potassium contents were estimated to be 179 and 162 mmol/3000 kcal, respectively. We observed that, while on the diets, the ratio of urinary potassium to sodium (K/Na) excretion increased by  $0.6 \pm 0.3$  in the ADA group and by  $2.0 \pm 0.8$  in the Paleo group. As expected, patients on the Paleo diet had greater decreases in the urinary sodium levels and increase in potassium levels and an increase in K/Na ratio compared with the ADA group. Calculation of potassium to sodium ratio confirmed that all the patients, except for one, on the Paleo diet were compliant with the diet. In the Paleo diet group but not the ADA group, there was a decline in urinary calcium/creatinine ratio by  $45 \pm 43$  (mg/g) and an increase in urine pH by  $+0.8 \pm 0.5$ . The Paleo diet was significantly different compared with the ADA diet group in terms of causing changes in urine pH and urine calcium excretion. (Table 3)

Lipid control. Baseline lipid concentrations are listed in Table 2. There were statistically significant reductions in total cholesterol, HDL cholesterol and low-density lipoprotein (LDL) cholesterol on the Paleo diet (Table 3). The total cholesterol, HDL cholesterol and LDL cholesterol trended downward on the ADA diet, but only the decline in HDL cholesterol reached statistical significance. The triglycerides trended downward to a greater degree on the Paleo diet than on the ADA diet.

Glucose control and changes in insulin resistance. The patients remained on the same medications for the duration of the study. In the Paleo group, the HbA1c declined by 0.3% over the 3 weeks of the study ( $P=0.04$ ) and 0.2% in the ADA group ( $P=0.04$ ). Fructosamine, which is a shorter-term marker of glycemic control, declined by  $34 \mu\text{mol/l}$  in the Paleo group ( $P=0.01$ ) and only by  $3 \mu\text{mol/l}$  in the ADA group. The ADA group and Paleo group were not statistically different in terms of insulin resistance at baseline (M/LBM/I),  $6 \pm 1.8$  vs  $7.1 \pm 2.3$  ( $P=0.3$ ). After the diet, the mean change in M/LBM/I was  $1.0 \text{ mg/min/kg/mU}$  insulin ( $P=0.1$ ) in the ADA group and  $1.3$  in the Paleo group ( $P=0.09$ ). Weight change could not explain the mean change in insulin resistance. In a bivariate analysis with group

allocation and weight change as explanatory variables, the change in insulin resistance was independent of weight change (ADA group,  $P=0.7$ ; Paleo group,  $P=0.1$ ). We did observe that, within the Paleo diet subjects, those who were the most insulin resistant (M/LBM/I) at baseline had the greatest improvement in insulin resistance with the diet ( $\Delta\text{M/LBM/I}$ ;  $r=0.63$ ,  $P=0.02$ ). In contrast, baseline M/LBM/I did not predict the response to the ADA diet (Figure 1).

**Blood pressure.** BP data are given in Table 3. The mean arterial pressure did not significantly change in any of the two groups—mean arterial pressure declined by  $-2\pm 7$  mmHg in the Paleo diet group ( $P=0.3$ ) and by  $-1\pm 7$  mmHg on the ADA diet.

**One-month follow-up data.** We also wanted to determine whether having been on an experimental diet had a sustained benefit, and hence we asked patients to return for metabolic testing 1 month after completing the diet protocol. At the end of the study period, subjects were sent out with information about their respective diets. Twenty-two of twenty-four subjects returned for the follow-up urine studies, lipid profile and euglycemic clamp. The urine studies indicated that patients had returned to their pre-existing diet in terms of sodium and potassium intake. Glucose control and lipid profiles reverted toward baseline in both groups (Figure 2).

**DISCUSSION** Obese patients with type 2 diabetes were randomly assigned to a Paleo-type diet or a standard diet based on the nutrition recommendations of the ADA. We observed greater effects on

Table 2. Baseline characteristics

ADA diet (n=10)

Paleo diet (n=14)

P value

Age (years)	56±13	58±8	0.7	BMI (kg/m <sup>2</sup> )	34 ±7	31 ±5	0.4	Systolic BP (mmHg)	125±11	121±16	0.5
Diastolic BP (mmHg)	68±7	68 ±9	0.9	MAP (mmHg)	87±7	86 ±10	0.7	Blood Fasting plasma glucose (mmol/l)	7.7±2.5	8.4±4.2	0.6
HbA1c (%)	7.0±1.5	7.3±2.1	0.8	Fructosamine (mg/dl)	264±49	294±108	0.4	Insulin sensitivity M/LBM/I	6.0 ±1.8	7.1±2.3	0.3
Total cholesterol (mg/dl)	176±50	192±52	0.4	Triglycerides (mg/dl)	149±73	149±75	0.9	HDL cholesterol (mg/dl)	46±13	51±12	0.3
LDL cholesterol (mg/dl)	92±40	114±41	0.2	Urine Creatinine clearance (ml/min/24h)	142±35	173±65	0.2	Urine K/Na mmol/mmol	0.4±0.2	0.5±0.3	0.3
Urine pH (U)	5.8±0.4	5.9±0.5	0.4	Urine Ca/Creat (mg/g)	91±29	93±23	0.8				

Abbreviations: ADA, American Diabetes Association; BMI, body mass index; BP, blood pressure; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MAP, mean arterial pressure.

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metabolic parameters while on the Paleo diet than on the ADA diet just after 3 weeks. The Paleo diet group had improvement in glucose levels—declines in HbA1c of 0.3% ( $P=0.04$ ) and fructosamine by 34  $\mu\text{mol/l}$  ( $P=0.009$ ). The ADA group had a 0.2% drop in HbA1c ( $P=0.04$ ) but no decline in fructosamine. This improvement in glycemic control with the Paleo-type diet could not be explained by changes in weight or by group improvements in insulin sensitivity—both diet groups had equivalent changes in

Table 3. Changes in blood and urine parameters within each diet and between diets

ADA P value for changes within ADA group

Paleo P value for changes within Paleo group

P value for differences between groups Weight (kg)  $-2.1 \pm 1.9$  0.004  $-2.4 \pm 0.7$  0.001 0.7Systolic BP (mmHg)  $-2 \pm 13$  0.7  $-4 \pm 12$  0.2 0.6 Diastolic BP (mmHg)  $0 \pm 12$  0.9  $-1 \pm 6$  0.4 0.6MAP (mmHg)  $-1 \pm 7$  0.8  $-2 \pm 7$  0.3 0.6Blood HbA1c (%)  $-0.18 \pm 0.24$  0.04  $-0.3 \pm 0.49$  0.04 0.5 Fasting plasma glucose (mmol/l) $+0.6 \pm 1.8$  0.4  $-1.3 \pm 1.4$  0.008 0.3 Fructosamine (mg/dl)  $-3 \pm 28$  0.7  $-34 \pm 41$  0.009 0.06Insulin sensitivity M/LBM/I  $+1.0 \pm 1.9$  0.1  $+1.3 \pm 2.6$  0.09 0.8 Total cholesterol (mg/dl) $-9 \pm 25$  0.2  $-26 \pm 27$  0.003 0.2 Triglycerides (mg/dl)  $-5 \pm 63$  0.8  $-23 \pm 46$  0.08 0.5 HDLcholesterol (mg/dl)  $-6 \pm 8$  0.03  $-8 \pm 7$  0.001 0.5 LDL cholesterol (mg/dl)  $-7 \pm 17$  0.2  $-15 \pm 22$ 

0.02 0.4

Urine CrCl  $-16 \pm 29$  0.1  $-3 \pm 29$  0.9 0.2 Urine K/Na  $+0.6 \pm 0.3$  0.0001  $+2.0 \pm 0.8$  0.0001 0.001Urine pH  $0.1 \pm 0.3$  0.7  $+0.8 \pm 0.5$  0.0001 0.001 Urine Ca/Creat  $-2 \pm 33$  0.9  $-45 \pm 43$  0.002

0.008

Abbreviations: ADA, American Diabetes Association; BMI, body mass index; BP, blood pressure; CrCl, creatinine clearance; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MAP, mean arterial pressure.

-6 -4 -2 0 2 4 6 8

0 5 10 15

-3 -2 -1 0 1 2 3 4 5

0 2 4 6 8 10

Pretreatment M/LBM/I

ADA,  $r=0.39$ ,  $p=0.3$ Paleo,  $r=0.40$ ,  $p=0.02$ M/LBM/I  $\Delta$ 

Figure 1. Scatterplot of change in insulin sensitivity as measured by euglycemic hyperinsulinemic clamp (change in mg glucose/min/kg/ mU insulin,  $\Delta$ M/LBM/I) against baseline M/LBM/I. The most insulin-resistant subjects had a significant improvement in insulin sensitivity on the Paleo diet ( $r=0.40$ ,  $P=0.02$ ) but not on the ADA diet ( $r=0.39$ ,  $P=0.3$ ).

6.3 6.4 6.5 6.6 6.7 6.8 6.9 7 7.1

PRE RX POST

140

150

160

170

180

190

200

PRE RX POST

ADA PALEO

Total cholesterol, mg/dL  $\Delta$  Hemoglobin A1c, %  $\Delta$ 

Figure 2. Metabolic testing was performed immediately before and after the diets and then 1 month after completion of test diets. The glucose and lipid changes were not sustained and reverted toward baseline in both groups at the 1-month time-point.

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weight and insulin sensitivity. In a bivariate analysis with group allocation and weight change as explanatory variables, the change in insulin resistance was independent of weight loss (ADA group  $P=0.7$ ; Paleo group  $P=0.1$ ). We did observe, however, that those subjects who were the most insulin resistant at baseline demonstrated the greatest improvements in insulin sensitivity on the Paleo diet but not on the ADA diet. This is similar to the effect we saw in our previous study of healthy sedentary volunteers; those who were most insulin resistant at baseline, using the HOMA index,  $((\text{fasting insulin} \times \text{fasting glucose})/22.4)$  demonstrated the greatest improvement in insulin sensitivity on the Paleo diet.<sup>10</sup> Our Paleo diet was not low in carbohydrates, but the sources of carbohydrates were different—from fruits, vegetables and honey. The ADA group in contrast ate rice, bread and pasta. The Paleo compared with the ADA diet was also high in fiber—about 35g/2500kcal vs 12g/2500kcal, and it is possible that the fiber attenuated the post-prandial glucose rise and that this was the main driver improving overall glucose control.<sup>18,19</sup> The Paleo diet group had statistically significant declines in total cholesterol, HDL cholesterol and calculated LDL cholesterol. The triglycerides trended down but did not reach statistical significance. In contrast, the ADA group only had a decline in HDL cholesterol but not in total cholesterol, calculated LDL cholesterol or triglycerides. Our Paleo diet was lower in saturated fats and higher in mono- and polyunsaturated fats compared with the ADA diet (Table 1), and it is likely that this explains the decline in the total and HDL cholesterol levels.<sup>20</sup> The modest decline in the HDL cholesterol on the ADA diet probably also reflects the improved fat composition of the experimental diet compared that which the patients were eating at home. There were no significant changes in systolic or mean arterial pressures with either diet. This is despite both diets being lower in sodium and higher in potassium than the baseline diets consumed by the subjects. We might have expected, based on our previous study<sup>10</sup> and published studies,<sup>13,14</sup> to see a more marked within-group effect of the Paleo diet or between-group effect with the ADA diet on the described metabolic variables. A number of factors, however, may have attenuated these effects. First, the patients overall were well controlled at baseline with HbA1c levels around 7%, systolic pressures in the mid 120s and triglycerides around 150mg/dl, and we kept them on their baseline glucose lowering, antihypertensives and lipid-lowering medicines. We might have seen greater benefits in patients who were not on treatment and less well controlled at baseline. Second, we wanted to evaluate the impact of the diets in the absence of weight loss, and even though we adjusted the caloric intake to avoid weight loss both the control and test groups lost ~2kg in weight. We might have seen more marked differences between the diets if there had not been any weight loss. The subjects on the Paleo-type diet did complain that the volume of food that they had to eat was excessive and without our encouragement would likely have lost more weight. Increased satiety has been reported on Paleo-type diets compared with Mediterranean diets.<sup>21</sup> Third, on the basis of our intake questionnaire and food recall responses, we expected the parameters of the subjects on the ADA recommended diet to remain unchanged from baseline, but in fact they also improved reducing the differences observed with the two diets. This may reflect the poor nutritional characteristics of the subjects' baseline diet and/or the bias effects of

being in a non-blinded clinical study. The metabolic benefits of the diets were not sustained, and both diet groups reverted back toward baseline 1 month after completing the diets. The study, however, was of short duration, and a definitive result on whether the Paleo diet can have sustained metabolic benefits would probably require a prolonged intervention. In conclusion, we demonstrate in a small randomized, metabolically controlled diet study that patients with type 2 diabetes can benefit from being on a Paleolithic-type diet compared with a conventional diet based on nutritional recommendations of the ADA. The nutritional composition of a Paleo diet—high-fiber content, high antioxidants, high mono- and polyunsaturated fats, low sodium and high potassium—may be particularly beneficial in these patients, even if they are on medicines to control glucose levels, BP and lipids.

**CONFLICT OF INTEREST** All the authors were involved in the design of the experiment. UM, PS, MS, SS, AX and LF performed the experiments. UM and LF analyzed the data and wrote the manuscript. The authors declare no conflict of interest.

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### 29. What are the benefits of a Carnivore Diet?

- a. Excludes phytates, lectins, and oxalates for the most part
- b. Works like an elimination diet
- c. Includes phytonutrients from leafy green vegetables
- d. Both A and C
- e. Both A and B

### OUR INDUSTRY TODAY

Importance of Animal Products in the Human Diet ~

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**ABSTRACT** Increased interest in food, nutrition, and health has led to critical evaluation of the American diet by governmental, scientific, and consumer groups. The exact role of diet in health and disease continues to be debated. Over the years trends in consumption of foods and nutrients have developed. These issues are examined. Historically, animal products have played a key role in the overall health of Americans. The contributions of animal products to human nutrition are emphasized along with an introduction to the use of the concept of nutrient density.

**SITUATION ANALYSIS** There has been increasing interest in and concern for the relationship between environment and health. This interest has been voiced both by those in government and by consumers (7, 17, 21, 27). Diet, as an integral component of our environment which can be modified, has received much of this increased attention as preventive measures are formulated to improve our quality of life. For example, in 1977

the US Senate Select Committee on Nutrition and Human Needs published, and subsequently republished with revisions, "Dietary Goals for the United States" (29, 31). If followed closely, these proposed goals would lead to widespread changes in food selection and eating patterns for many Americans (23, 24, 26). Basically, the goals promote decreased consumption of

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fat, sugar, salt, and alcohol and increased consumption of more complex carbohydrates (breads and cereals, fruits and vegetables). The changes proposed should be evaluated closely for potential benefit. This is especially critical as the dietary goals may serve as a template for a broader national nutrition policy in the future. Members of the scientific and medical communities do not agree completely on the dietary goals, the way they were developed, or the expected benefits which might be derived from their adoption (10, 11, 12, 13, 14, 30). Indeed, this latter point is a major concern. As presented, the approach of the dietary goals may raise false hopes among consumers. If the promised results are not obtained, the public could be disillusioned with governmental nutrition and health programs and possibly even with the science of nutrition. Depending upon the reference (12, 13, 29), changes in the food supply during this century have been associated with improving or deteriorating health. But the predominance of evidence agrees with Leveille (13) when he suggested that today's American diet is one of the best in the world. During the past half century in the United States, nutritional deficiency diseases have been reduced greatly, most infectious diseases are controlled effectively, infant and child mortality have decreased steadily, and life expectancy has increased by 20 years. The American diet has had an impact on these trends, and animal products have been and continue to be a prominent part of the American diet. Animal products are indicted for their role in the major "killer diseases" of developed countries (coronary heart disease, cancer, stroke and hypertension, diabetes, and cirrhosis of the liver) (29). However, even critics of animal foods have to acknowledge that most of the nutrients consumed in the American diet in less than recommended amounts are provided

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by animal products. For example, calcium generally is not consumed in recommended amounts by the adult population, but 81% of the calcium in our diets is provided by animal products (75% from dairy products alone) (15).

WHAT DO STATISTICS REVEAL? As indicated earlier, the incidence of infectious diseases and diseases from nutritional deficiency is decreasing in the United States while the incidence of certain chronic diseases has been increasing in recent years, especially with the greater number of people living longer. Life expectancy has increased 53% in 75 yr (47.3 yr (1900) to 72.5 yr (1975)) (36). Trends in nutrition and diets in the United States have been reviewed (3, 6). While the available supply of total calories and calories derived from protein have remained fairly constant, the proportion of calories from fat has increased and the proportion of calories from carbohydrates has decreased. The decreased consumption (disappearance) of total carbohydrate also represented a shift in the kind of carbohydrate. Following the 1920's the use of refined sugar increased and has been maintained while the use of starch and more complex carbohydrates has continued to

decline. Total fat consumption (disappearance) has increased along with a change in the type of fat. There has been only a small change in saturated fatty acids in the food supply since the early 1900's, however, increased use of polyunsaturated fatty acids has been marked. Daily intake of dietary cholesterol has risen only about 1% since the turn of the century. This is attributed largely to the increased total intake (disappearance) of meat and poultry products. However, use of specific products such as eggs, lard, and butter has declined during this period. Calcium, vitamin C, and vitamin A are more plentiful in our food supply than 65 yr ago. The enrichment of flour with iron, riboflavin, niacin, and thiamin markedly improved the availability of these nutrients since 1941. Early in this century about one-half of the protein came from animal products. In 1972 roughly 70% of the protein supply came from meat, poultry, fish, dairy products, and eggs. Long-term trends in consumption (disappearance) of major commodities are explained by trends of nutrients. Since 1909 amounts of cereal products have declined, and meat, poultry, and fish have increased. Dairy products (including butter) increased until the mid-40's and since have leveled off. Consumption (disappearance) of fruits and vegetables increased through the mid-40's and has decreased since. Gormer (6) examined the data for who is consuming the various food products. For instance, consumption (disappearance) of dairy products declines after age 8 for females but stays high for males through age 19. Apparent consumption of animal products does not change greatly for females from the teens through adult life; however, it continues to increase for men through age 34. To develop an effective national policy on food, nutrition, and health, the above mentioned changes in consumption of food and nutrients over time are relevant. However, it is also essential to have some idea of the nutritional and health status of the US population in relation to their nutrient needs. Surveys such as the USDA Household Food Consumption Survey of 1965 (33), the HEW Ten-State Nutrition Survey of 1968 to 1970 (34), and the HEW Health and Nutrition Examination Survey (HANES) (35) reveal that in a society as affluent as the United States there still exist populations with inadequate nutrient intake. The nutrients most frequently identified as being consumed in marginal or in less than recommended amounts are iron, calcium, vitamin A, riboflavin, and vitamin C. Animal foods are excellent sources of four of these nutrients (iron, calcium, vitamin A, and riboflavin).

**NUTRIENT CONTRIBUTIONS** See Table 1 for a summary of the contributions of meat, eggs, and dairy products to available nutrient supplies. In comparison to other food groupings (15), animal foods are excellent sources of many of the nutrients. One notable example is protein (meat, eggs, and dairy products provide about 69% of the available supply). Animal protein is higher in nutritional quality than is plant protein, because the assortment and amount of amino acids in animal protein more closely matches needs of the human body. Animal products can be used to upgrade the nutritional quality of plant sources of food by

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TABLE 1. Contribution of meat, eggs, and dairy products to the food supply (1978).a

Nutrient Meat b Eggs Dairy c Total

(% of total)	Food energy (kcal)	19.8	1.8	11.2	32.8	Protein	42.3	4.8	22.1	69.2	Fat	33.6	2.8	12.5	48.9	Carbohydrate	1.1	6.7	6.9	Calcium	3.9	2.2	74.6	80.7	Phosphorus	28.3	5.0	35.0	68.3	Iron	30.6	4.7	2.5	37.8	Magnesium	14.2	1.2	21.7	37.1	Vitamin A value	22.8	5.7	13.3	41.8
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Thiamin 26.0 2.0 8.7 36.7 Riboflavin 24.4 4.6 39.3 68.3 Niacin 45.1 .1 1.4 46.6 Vitamin B 6  
47.4 1.8 10.5 59.7 Vitamin B 12 70.6 7.8 20.1 98.5 Ascorbic acid 1.1 .0 3.8 4.9

aFrom USDA (15). bMeat (including pork fat cuts), poultry, and fish. cDairy products, excluding butter.

virtue of this high quality protein and also due to excellent B-vitamin content and iron in highly available form. Proteins of grains are generally low in lysine, and corn is low in tryptophan while milk and meat are good sources of lysine and tryptophan. Cereal grains are low in calcium while milk is high in calcium. The efficiency of animals in producing human food must be evaluated in terms of nutrient needs (18, 25). For instance, ruminants, by utilizing roughages, can be noncompetitive with man for food supplies. In addition, the ruminant produces a high quality food product (i.e., meat and milk). By utilizing animal protein sources to meet a portion of the human needs for protein, less total dietary protein would be required than if plant foods were the sole source of protein.

**HEALTH CONCERNS** A number of conditions of health and disease have been associated with the presence or absence of animal foods in the diet of populations (29, 30). These data, however, have little relevance to individuals within such populations. For these conditions, the evidence should be examined to put the entire issue in perspective. Some people are at greater risk of developing certain disease than other people. A dietary treatment for a certain disease may benefit some individuals but not the entire population. Nutrients, interactions among and between nutrients, and other components of the diet most likely have a greater effect on specific conditions of health and disease than do specific foods. For this reason it is misleading to suggest that any single food is good or bad relative to certain health conditions. Examples of health concerns often associated with consumption of animal products are coronary heart disease, cancer, obesity, iron-deficiency anemia, and bone disease.

**Coronary Heart Disease (CHD)** The main discussion in CHD centers around the amount and kind of dietary fat and the relationship of this diet component to serum cholesterol (a risk factor for CHD). The effectiveness of dietary change alone to control CHD is not agreed upon by all scientists (30). Several epidemiological studies within homogenous populations have failed to demonstrate significant associations between individual nutrient intakes and either blood lipids or CHD risk within that population (5). While some drug treatments may lower cholesterol in serum modestly and reduce the incidence of nonfatal heart attacks in subjects with initially high serum cholesterol, the effectiveness of this lowering on mortality from CHD was not demonstrated (22). Likewise, the use of dietary manipulations to decrease cholesterol in blood and in turn delay or prevent the incidence of CHD, remains an area of active research. As an example, the National Heart, Lung, and Blood Institute currently is supporting large clinical trials aimed at evaluating the effect of diet and other variables on the primary risk factors in CHD. One of the questions as yet unanswered is whether specific lowering of blood cholesterol through changes in diet of the average American will prevent CHD (28). If one compares the amounts of fat provided by animal and vegetable sources in 1909 and 1978 (Table 2), total dietary fat has increased due largely to increased consumption of vegetable fat (15).

**Cancer** In recent years the relationship of diet to

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TABLE 2. Fat provided by animal and vegetable sources, a

Animal Vegetable Total Year fat fat fat

-- (g/capita/day)- 1909 to 1913 103.5 21.3 124.8 1978 91.2 67.9 159.1

aFrom USDA (15).

various forms of cancer has been examined (2, 20). Almost no food has been exempt from examination since diet represents an environmental factor which can be modified in studies of cancer. Generally, diet is believed to influence organ susceptibility and response to other causative cancer factors (2, 20). At this point, various foods or nutrients are not believed to be carcinogens themselves. Epidemiological studies show incidence of certain forms of cancer correlates with diets high in animal foods such as meat. However, these same diets tend to be low in fiber. It is conceivable that what the diet does not contain could be just as critical as what it does contain. To date, most studies on diet and cancer have been with animals, and results often have been conflicting (2). For instance, a given nutrient in various amounts may exhibit both protective and enhancing effects on certain cancers. Future research with selected populations (such as Mormons and Seventh-Day Adventists) should offer unique opportunities to examine the relationship of diet and nutrition to cancer. These groups have lower than average incidence for many cancers; however, their consumption of various animal products often equals or exceeds the national average.

exercise). In controlling calorie intake the question is often where to cut back on calories in the diet. The wisest choice is to consider sources of nutrients in relation to calories. Animal products provide many essential nutrients in relatively large amounts in relation to total calories provided. Thus, animal products have high nutrient density. On the other hand, fats, oils, sugars, and sweeteners provide a large proportion of total calories but few additional nutrients. These foods would have low nutrient density. Thus, to control obesity, intake of low nutrient-dense foods should be decreased.

Iron-Deficiency Anemia Surveys have indicated an inadequate iron intake by some segments of the population (young children and young women) (33, 34, 35). Animal products such as meat are excellent sources of dietary iron in a form which has high bioavailability (16).

Bone Disease Decreased density of skeletal bone (osteoporosis) and alveolar bone (periodontal disease) in advancing age resulting in bone fractures indicate that calcium intake and/or dietary factors affecting bone development may be lacking (1). Or, there may be an excessive intake or altered ratio of factors adversely affecting bone integrity (32). Of all foods, dairy foods are the major dietary source of calcium (75% in 1978) (15). Relative to these latter two health concerns, recommendations to increase dairy food consumption for its bioavailable calcium and to increase meat consumption for its bioavailable iron would go far in fulfilling our inadequate or marginal nutritional status of these nutrients. Obesity The trend to overconsumption and decreased physical activity has made obesity the most widespread nutritional problem in the US. This condition also represents the common denominator of many nutrition-related public health problems (30). It is a risk factor in CHD, cancer, hypertension, atherosclerosis, diabetes, arthritis, and other diseases. To control body weight one must control excessive calorie intake and/or increase caloric expenditure (i.e.,

NUTRITION EDUCATION Since 1943 the "Recommended Dietary Allowances" (RDA) established by the Food and Nutrition Board, NAS-NRC, have provided what might be considered national nutritional guidelines. These nutrient allowances are revised

periodically (9th edition to be published in 1979). The RDA are intakes of essential nutrients adequate to meet the known nutritional needs of practically all healthy persons in the US (4). Over the years they have served

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as valuable national guidelines for nutrition upon which to base successful programs of nutrition education. Many years ago USDA and National Dairy Council (NDC) started to group foods with similar nutrient content to facilitate teaching nutrition (19). The major groupings include milk, meat, fruit-vegetable, and grain. Foods not included in these four food groups supply few nutrients except carbohydrate and fat and, thus, are categorized as "others". They complement but do not replace foods from the four food groups. The recommended number of servings from the four food groups is designed to meet the RDA for most nutrients with calories consistent with desirable body weight. A daily dietary pattern meeting the RDA for established nutrients usually can be achieved with about 1200 kcal. If needed, additional energy can be obtained from more servings from the four food groups or servings from "others". The food grouping system provides a simple and effective way to encourage recommended servings of a wide variety of foods to achieve a nutritionally adequate diet.

**NUTRIENT DENSITY** An important concept in formulating diets is to obtain the recommended nutrients in adequate amounts with a minimum intake of calories. To do this one would choose foods with a high proportion of nutrients to calories, nutrient-dense foods. As was mentioned earlier, animal foods are some of the most nutrient-dense foods. However, no one group of foods represents the best source of all nutrients. Thus, variety in the diet is essential.

Nutritionists are working continually to devise improved methods for evaluating nutrient contributions of various foods. The concept of nutrient density has received increased attention (8, 9). The Index of Nutritional Quality (INQ) as reported by Hansen and colleagues represents one approach to utilizing nutrient density. The INQ of a food for a particular nutrient is the ratio of the percent of the nutrient allowance to the percent of the energy allowance provided by a serving of the food (Table 3). The INQ is useful in determining the relative adequacy of a food as a source of a certain nutrient. For instance, an  $INQ > 1$  indicates that an amount of the food which would supply the total energy allowance would supply more than the recommended amount of that nutrient. A 4 means that the recommended amount for that nutrient would be met at 25% of the total energy allowance from that food. This is an example of how nutrient density can be considered in constructing calorie-restricted diets. Conversely, an  $INQ < 1$  indicates that a food is not a major source of the nutrient since consumption of the food in excess of caloric recommendations would be needed to meet the recommended allowance. The INQ, while being a significant step forward, does need further refinement. For example, bioavailability of nutrients and amount of food needed are not considered. Table 4 shows the INQ for several animal products for the nutrients required on nutrition labeling.

TABLE 3. Calculation of Index of Nutritional Quality (INQ).a

INQ

% of nutrient allowance / % of energy allowance

Example: Allowances -- 2300 kcal and U.S. RDA for nutrients

Whole milk

Calcium (ca) INQ  
 Milk Ca/USRDA Ca  
 =  
 Milk kcal/kcal RDA  
 Milk C/USRDA C  
 =  
 Milk kcal/kcal RDA  
 Vitamin C (C) INQ  
 291 rag/1000 mg  
 = .29/.065 = 4.5  
 150 kcal/2300 kcal  
 2.29 mg/60 mg  
 = .04/.065 = .6  
 150 kcal/2300 kcal

aAdapted from Hansen et al. (8).  
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TABLE 4. Index of Nutritional Quality (INQ). a  
 Skim Plain Cheddar Ice Hamburger Pork Beef Nutrient Milk milk yogurt cheese cream (very lean) Bacon loin liver  
 Protein 1.9 3.6 3.0 2.2 .7 4.4 1.6 4.0 4.0 Vitamin A Act. 1.0 2.6 .5 1.2 .9 .1 ..... 107.1 Vitamin C  
 .5 .9 .5 .... 1 ..... 4.5 Thiamin .9 1.7 1.1 .1 .3 .7 1.4 6.4 1.7 Riboflavin 3.6 6.5 4.6 1.3 1.7 1.5 .8  
 1.6 24.7 Niacin .2 .3 .2 .1 3.2 1.1 2.9 8.2 Calcium 4.5 8.2 6.7 4[2 1.5 .1 .1 .1 .1 Iron .1 .1 .2 .2 .1  
 2.1 .8 1.9 4.9

aBased on U.S. RDA, 2300 kcal energy requirement and USDA - HGB #72, 1977 nutrient data.

FUTURE NEEDS

Although animal products currently represent excellent sources of nutrients, they can be improved. As with any natural foodstuff, many beneficial nutrient interactions still undiscovered potentially exist among animal products and between animal products and other foods. Further research should document these benefits for human health. The animal industry should be aware of consumer needs and wants. For instance, fortified or modified products may be desired for certain populations. There continues to be a tremendous need for effective nutrition education which stresses caloric moderation and emphasizes consumption of a variety of foods.

CONCLUSION Animal products occupy a unique position in human nutrition. They are nutrient dense and represent convenient sources of highly bioavailable nutrients. The overall quality and quantity of their nutrient profile upgrade the lower quality of foods from plants. Animal products are fundamental to the achievement of a nutritionally adequate diet. They have contributed in a major way to our present quality of life and nutritional well-being

What to Eat on a Carnivore Diet. Your Carnivore Diet Meal Plan! Written by Paul Saladino on August 7, 2019

You've heard the stories of people finding significant improvements on a carnivore diet with everything from weight loss, depression and anxiety, to autoimmune disease, and you want to give it a try! Well, this is the post for you. In this post I'll talk about a few variations of the carnivore diet, and include some sample carnivore diet meal plans to help you get started. The first thing you need to ask yourself is what your goals are for this way of eating, and what fits your lifestyle. For the purposes of this article, I'll divide the carnivore diet into 5 different tiers. Based on your goals you can decide which tier is right for you. I talk about this in much more detail in my upcoming book, "The Carnivore Code: Unlocking the Secrets to Optimal Health by Returning to Our Ancestral Diet."

One other consideration in the discussion of what to eat on a carnivore diet is WHEN to eat. I'll do a whole separate blog post about intermittent fasting and time restricted eating. Here's the short version: because a carnivore diet is so satiating, most people find that eating two times a day, or even once per day (known as OMAD) works better than three meals per day. This also makes time restricted eating much easier by allowing for a more compressed eating window with less meals. In the meal plans that follow, I have suggested breakfast, lunch, and dinner meals, but twice or once per day eating is totally appropriate, and perhaps even better! The Tiers

Tier 1: Carnivore ISH (with discussion of low and high toxicity plant foods)

Also known as "carnivore adjacent", this type of eating emphasizes animal foods, and consumes these as the majority of the diet, but allows some room for what I would consider the least toxic plant foods. Beginning with an appreciation of the fact that animal foods represent the most nutrient rich sources of bioavailable vitamins and minerals, these foods form the majority of such a diet, perhaps 80%-90%. These foods might include ruminant (beef, bison, lamb) meat, poultry, fish, eggs and dairy for those who tolerate (see discussion below for more information on this topic). In addition to these foods, "low toxicity" plant foods may be included for flavor, preference or texture/color. I will reiterate here that I see plant foods as "survival foods" and don't believe they provide unique nutrients for humans that we can't obtain from animals. Furthermore, plants have lots of toxins in them, many of which have been misconstrued as beneficial for humans, that irritate the gut and the immune system.

I see a whole foods animal based diet as the most basic diet that humans will thrive on. There does appear to be some genetic variability in human response to ratios of animal fats, however, and there are rare examples of individuals who do not oxidize fats for fuel well. For the VAST majority of people, however, an animal based diet is an ideal foundation. Genetic variability also

appears to come into play regarding which plant foods a given individual will tolerate. In some individuals, any amount of plant foods and dairy appear to trigger the immune system, leading to resurgence of inflammation and autoimmunity. In others, specific plant foods may be tolerated without apparent detrimental effects. This is an individual idiosyncrasy, and will need to be determined on such a basis. For the purposes of this blog post, however, I will discuss a few foods that might be considered to be the "least toxic," and that could be added to a "carnivoreish" diet. The understanding here, of course, will be that much of this will be unique based on the individual.

Which are the least toxic plant foods? I generally think of these as the non-sweet fruits, and include things like olives, avocado, lettuces, cucumbers (without skin or seeds), and various squashes in this group. Squash, in particular, will have higher amounts of carbohydrates, and will interrupt efforts toward ketosis if that's a goal. On the flip side, for those interested in incorporating carbohydrates into their diets prior to long, intense athletic efforts, squash might be a good option for this. Removal of skin and seeds of the squash would likely decrease lectins significantly in this case.

What about more toxic foods? At the opposite end of the spectrum I would place plant seeds. The category of plant seeds really includes seeds, grains, nuts, and beans. These are all plant seeds, and they are all very heavily defended by plants. They contain digestive enzyme inhibitors, lectins, high amounts of phytic acid- a molecule that binds phosphorus in plants, but can also bind other positively charged ions such as Mg, Zn, Ca, and Se, limiting their absorption. In addition to plant seeds, the nightshade or solanaceae family (tomatoes, eggplants, potatoes, goji berries, peppers, paprika, chili peppers) is also known to be a common immune trigger.

Most fruits and vegetables lie between these two groups, and are difficult to qualify in terms toxicity. This will vary on a person to person basis. On a tier 1 Carnivore diet, you might start with the low toxicity plants and add in moderate toxicity foods to see how you tolerate them. Many people will do best with NO plants for some amount of time, however. When all plants are eliminated, we move to a Tier 2 carnivore diet.

Beverages: Many people ask about coffee. I'll do a whole separate post on this topic. The short answer is that I am not a fan of coffee for a variety of reasons including caffeine (sympathetic nervous system activation, sleep disturbance, etc.), pesticides, mold toxins and acrylamide formed in the roasting process. If you choose to include coffee in your diet, know that my general experience is that most people feel much better without it once they have gone through the acute withdrawal phase. In place of coffee, I generally recommend high quality, purified water. Distilled water or Reverse Osmosis should be remineralized but is a good option. High quality carbon filters like the Berkey (no affiliation) are another option. The ideal water would be locally sourced spring water ([findspring.com](http://findspring.com)), but this is not always available. Sparkling mineral water is fine – I'm a big fan of Gerolsteiner, which is particularly rich in minerals, and Topo Chico. Tea contains many compounds, like tannins, which may impair nutrient absorption and irritate the gut. I generally recommend against it. Sodas, fruit juices, etc. are clearly not ideal and should be avoided.

What a typical day of Tier 1 Carnivore looks like:

(All "typical days" will vary based on your goals, body composition and metabolic rate. Twice per day or once per day eating styles are also appropriate).  
 Breakfast: • 3 scrambled eggs with 1 tablespoon of ghee • 1/2 Avocado with Sea salt  
 Lunch: • 6oz grass fed ribeye steak • Cucumber slices and romaine lettuce with olive oil dressing  
 Dinner: • 8oz lamb chops • Olives • 1/2 avocado

Tier 2: Meat/Water

This is the most basic, and simplest version of a true carnivore diet. It's for people who want to experiment with a whole foods animal based diet for short amounts of time, like an elimination diet. In my opinion, this type of carnivore eating is not ideal for most people for the long term, but it could serve as a very simple introduction to this way of eating. On a Tier 2 Carnivore diet "eat meat, drink water" is the classic adage which describes this way of eating best. It's a pretty simple formula, and as an elimination diet it can be a very helpful

tool. My concerns with this type of diet long term are nutrient deficiencies. I did a podcast with Amber O'Hearn in which we talked about nuances regarding RDAs on a carnivore diet. It's pretty clear that our body's requirements for many things changes in the absence of carbohydrates. Even meat has a small amount of carbohydrates but for the purposes of this discussion, they are essentially negligible. There's a whole facebook group (Zeroing in on Health) dedicated to this type of diet, and there are many examples of people who appear to thrive eating only animal meat and drinking water. Examples include Joe and Charlene Andersen, and Charles Washington, who moderates the aforementioned facebook group.

While I do think a tier 2 carnivore diet can be very helpful for some people, adding even a few foods like eggs and occasional seafood can help fill in many of the potential nutrient gaps. Evolutionarily, I also don't think that we would have only eaten the muscle meat of animals. There are numerous examples from anthropological literature to suggest that many indigenous peoples actually favored organ meats and fat, and ate muscle meat last, or even fed the muscle meat to the dogs. We'll talk more about adding organ meats in Tier 4/5 carnivore diets, but first let's talk about adding just a few more basic nutrient rich animals foods to the meat and water diet.

What a typical day of Tier 2 Carnivore looks like:

(All "typical days" will vary based on your goals, body composition and metabolic rate).

Breakfast: • 10oz grass fed ribeye steak with sea salt Lunch:

- 8oz lambburgers with sea salt Dinner:
- 8 oz grass fed NY steak

Tier 3: Basic Carnivore diet The basic carnivore diet adds a few things to the Tier 2 Meat and Water plan. This where most folks start out, and then usually progress to Tiers 4 and 5 as they get more excited about eating organ meats. The Tier 3 meal plan includes meat, eggs, seafood, and dairy, if tolerated.

A few words about dairy: I've personally found that all types of dairy trigger my eczema, and in many clients I work with, exclusion of dairy allows for increased satiety, less inflammation, and easier weight loss. Generally speaking, I do feel that dairy can be triggering for many people. If you have an autoimmune issue or you are really interested in losing weight, I'd leave dairy out for at least the first 60 days of a Carnivore diet. There's a bit a nuance here as well with regard to A1 vs A2 variants of casein, which breaks down into beta casomorphin. The name of that molecule looks like "morphine," and it acts in a similar, though much less intense, way in the human body by activating opioid signaling pathways. I'll do a whole separate blog post about A1 vs A2 dairy. The cliff notes version is that casein has two variants (gene polymorphisms, or SNPs), A1 and A2, which are broken down into different forms of beta- casomorphin.

The A1 variant of casein becomes beta-casomorphin 7, a molecule that has been linked (1,2,3) to increased incidence of autoimmune disease and cardiovascular disease. The take home message here is that if you're going to do dairy and don't think it triggers your immune system, opt for A2 dairy rather than A1. All non-cow dairy including goat, sheep, and buffalo are considered A2 dairy. There are bovine species which are A2, like many Guernsey cows, but these will be noted on the labeling and are much more rare. If it's from a cow, and doesn't specify A2 on the label, assume it's A1 dairy.

What a typical day of Tier 3 Carnivore looks like: Breakfast: • 2 eggs cooked in tallow or ghee with bacon

- 4 oz striploin steak Lunch:
  - 3 oz king salmon with butter/ghee Dinner: • 6oz shrimp • 8oz grass fed ribeye steak with sea salt
- Tier 4: Junior varsity organ meat eating and real animal fats This tier is for you if you are loving the carnivore diet and are organ-curious. You've heard me extoll the virtues of organ meats, like liver, and would like to incorporate this into your diet. You've also heard me talk about the amazing virtues of grass-fed fat trimmings or suet (beef kidney fat) and you'd like to jump on the fat train. A tier 4 carnivore diet will probably suit your needs very well, and I believe that you will notice improvements in mental clarity, satiety, and athletic performance by upgrading your diet in these ways.

Let's start with liver! Isn't this organ the body's filter and full of toxins? Nope! It is true that the liver contains the majority of the enzymatic systems involved in detoxification. These are referred to as the phase 1 and phase 2 detox pathways. The liver doesn't store toxins, however. It chemically transforms them with these systems to prepare the toxins for EXCRETION in the urine and feces. This is how we get rid of the bad stuff – we don't want nasty chemicals and compounds hanging around our bodies. If you've heard me talk about phytochemicals like sulforaphane or curcumin, you'll know that these compounds are detoxified in phase 1/2 and then excreted. Yes, I did indeed call these compounds toxins, and I don't feel they have any beneficial place in human nutrition. You can find much more in depth discussions of plant toxins on many of the podcasts I've been on. See the post on which podcasts I've been on [here]. Particularly in depth episode include Peak Human and Ben Greenfield.

So the liver isn't a filter, you get it, but you didn't grow up eating liver and the taste is different than what you are used to... Is it really that uniquely nutritious? In a word, yes! Muscle meat from animals is very rich in a lot of vitamins and minerals, but it doesn't have all of them. Simply adding liver to a Tier 3 carnivore diet really helps fill in many of the possible nutrients that could be limited on this type of diet. Granted, eating eggs and seafood will provide more nutrients than a meat and water diet, but I think adding in liver will be even better.

What nutrients am I talking about here? Liver is particularly rich in a few minerals and B vitamins which complement those found in muscle meat. On the mineral side, liver is one of the best sources of copper, which we need for enzymes like Super Oxide Dismutase (SOD). SOD serves a critical role in the antioxidant management system in our bodies (I talk about this in the podcast I did with Dom D'Agostino, PhD) by converting the superoxide radical (O<sub>2</sub><sup>-</sup>) into molecular oxygen (O<sub>2</sub>) or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Copper deficiency would result in accumulation of O<sub>2</sub><sup>-</sup>, which could have disastrous consequences in terms of excess oxidative

stress. Copper deficiency is rare, but it can occur if we consume too much zinc without some copper to balance it out. The most common situation for this to occur would be excess use of zinc supplements without a good source of copper in our diets, but it is also possible through diet if we get a lot of zinc in muscle meat without some source of copper. Clinical copper deficiency manifests with neurologic symptoms which mimic B12 deficiency (difficulty with balance, walking). Yikes! No fun!

Aside from copper, liver is also a great source of MANY other minerals including iron, selenium, manganese, and molybdenum. It's also very rich in choline, which has been unjustly maligned in connection with TMAO (see the podcast I did with Tommy Wood, PhD

in which we discuss this), and is a vital nutrient for healthy cell membranes and neurotransmitter production.

Looking at the B-vitamins, liver is just a powerhouse, on the order of the Incredible Hulk or another Avengers superhero. It provides significantly higher levels of almost all of these nutrients, and is an especially good source of folate, biotin, and riboflavin, which really are not that available in muscle meat. If you have an MTHFR or PEMT polymorphism (check out the podcast with Dr. Ben Lynch) you'll require more riboflavin than the general population, and liver is just about the richest source there is! Other good sources of riboflavin are heart, kidney, and egg yolks, with muscle meat having some but not nearly as much as these special foods. If you are interested in eating heart and kidney, you are probably ready for a Tier 5 Carnivore diet!

What a typical day of Tier 4 Carnivore looks like: Breakfast: • 2eggs • 4oz tenderloin steak • 2 oz liver Lunch: • 3oz grass fed suet • 8 oysters • 3 oz king salmon Dinner: • 6oz shrimp • 8oz grass-fed ribeye steak with sea salt Tier 5: Optimal Nose to Tail Carnivore diet (varsity organ meats) You are focused on optimizing your diet for best results in terms of resolution of inflammatory issues, weight loss, or physical/mental performance, and you want the Ferrari version of the carnivore diet. This is it! Tier 5 is basically how I eat day in and day out. This is the ultimate Carnivore MD diet. As I've noted earlier, this type of a carnivore diet may not be for everyone at

all times. Traveling makes eating lots of high quality animal meats, organs and fat difficult from time to time, and I get this. It's totally ok to use Tier 1-4 diets in your life when they are the most appropriate for your current situation. In these situations, desiccated organ supplements can help make incorporating organ meats in your diet more convenient. So how do I eat, and what do I think is the BEST way to construct a nose to tail carnivore diet? There are a couple of pieces to this equation. The first thing I think of is the fat to protein ratio in terms of macronutrients. I'll do a whole separate post on this. If you want to listen to me discuss the pros/cons of high fat vs. high protein with Ted Naiman, check out the Better, Stronger, Faster podcast I did with him here. I will also post a link to my Ancestral Health symposium talk on the unique nutrient value of animal fat once it's posted.

My general perspective is that animal fat is a vital and valuable part of animals that humans have uniquely sought out throughout our existence, and which should not be neglected or undervalued. Just like how liver and the other organ meats have a unique nutritional profile, animal fat does as well and I believe that it should be intentionally included on a well constructed nose to tail carnivore diet. If you are eating grass-fed meat there's some fat with cuts like ribeye and NY strip, but it's not a ton. Most of the fat is now trimmed off our meat by butchers, so we have to specifically ask for trimmings or look for the fat around the kidneys (suet). Grain-fed meat is certainly fattier but as I talk about in this blog post, I have some concerns about grain-fed fat accumulating more toxins like estrogen mimicking compounds, pesticides and dioxins. Personally, I source grass-fed suet from US Wellness Meats or White Oak Pastures (no affiliation) and include this as a large component of my diet. How much fat do I eat? Since I am at my goal body weight and composition, I am most interested in athletic performance. With this in mind, I aim for about 1.5-2g of fat per gram of protein that I eat on a daily basis. For protein, I aim for about .8g per lb of lean body weight per day. As a 170 lb dude, this ends up being about

140g of protein and 230-280g of fat per day! Are these macros causing me to lose lean mass or accumulate fat? I'd say definitely not, but I'll let you be the judge.

All of this grass-fed animal fat I am consuming is a source of unique nutrients. What?! Fat has nutrients? You bet it does! Grass fed animal fat is a great source of fat soluble vitamins like vitamin E and vitamin K2. In the Rotterdam Study, increased consumption of vitamin K2, but not K1 (from plants) was associated with significantly improved coronary heart disease outcomes. Grass-fed animal fat is also a source of the omega-3 fatty acids, EPA, DHA and DPA. My levels of omega-3 are robust eating a tier 5 carnivore diet.

I also test my micronutrients, including vitamin E, regularly. The results below are from July 2019. As you can see, my CoQ10 is off the charts (this is common in clients I work with on the carnivore diet), and my B vitamin markers are all looking great. My homocysteine is 7, which is exactly where I would like to see it. Interestingly, I am homozygous for the 677C->T polymorphism of MTHFR, and I don't supplement with any methylfolate. It's clear from this lab result that I am getting enough riboflavin from the liver I eat. The folate in liver is also L-5 methylfolate, rather than dihydrofolate, as is found in plants. Check out the podcast I did with Dr. Ben Lynch for a full discussion of these polymorphisms. I'll also do a whole podcast and separate post about all of my bloodwork eventually.

Looking at this section of my bloodwork, notice how high my vitamin E is. It's actually above the range True Health lists as normal, but this isn't a bad thing. I don't supplement with any sort of vitamin E. This is exclusively coming from grass-fed animal fat! One of the critiques that has been leveled against the carnivore diet is that this diet could be low in vitamin E. My results, and the results of my clients, would argue strongly against this. Check out the whole podcast I did answering common critiques of the carnivore diet if you'd like to dig deeper into all of this. A tier 5 carnivore diet also includes lots of organ meats. I personally favor these in my diet and usually end up eating a variety of them throughout the day. I do try to rotate the organ meats I eat throughout the week as I believe our ancestors would have. What I do may not work for everyone, and some of the organs I eat may be considered "gross" in terms of what is mainstream, but I find great value in making efforts to eat as much of the animal as I can. In a given week I will eat about 16 oz liver, 16 oz kidney, 16-32oz heart, 16oz testicles (yes!), and occasional spleen, pancreas, and brain when they are available.

You certainly don't have to eat all of these organs to do a great version of a tier 5 carnivore diet, but they are worth exploring. This may also be a place where desiccated organ capsules can help us get a wider variety of organs. In the section below about a typical tier 5 diet I will describe what I eat so people can get a sense of this. Again, just because I do it this way does not mean it's the only way to do it! The other disclaimer here is that while I eat many foods raw, this certainly presents contamination risks and it's not something I recommend unless you know the quality of your sourcing very well.

What a typical day of Tier 5 Carnivore looks like for me: Breakfast: I eat twice a day and usually don't eat breakfast. I opt for an early "lunch" instead. This usually happens around 10am. Lunch: • 6 raw egg yolks • 100g beef suet with sea salt • 2oz raw liver • 2oz kidney • 6oz ribeye steak Dinner: • 4oz testicle • 150g beef suet • 8 oz ribeye steak At this point you are probably saying, "Paul, you are crazy!" I've been called worse things! So people

have a sense of another version of a tier 5 carnivore diet, I will offer a “non-Paul” version below.

What a typical day of Tier 5 Carnivore looks like for someone: Breakfast: • 3 eggs • 2 oz beef liver • 1 oz kidney • 6 oz NY steak Lunch:

• 100g beef suet • 4oz scallops cooked in tallow Dinner: • 100g beef suet • 8 oz ribeye steak • 6 jumbo shrimp

The Carnivore Diet as an Elimination Diet The Carnivore Diet as an Elimination Diet Some people regard the Carnivore Diet as an Elimination Diet. An elimination diet excludes foods in an effort to identify allergies, intolerances, or other problematic foods.

Since the Carnivore Diet removes plant-based foods it removes nearly all potential offenders including problematic lectins, oxalates, alkaloids, salicylates, and many others.

In this regard, the Carnivore Diet is the ultimate elimination diet. It’s congruent with what we are designed to eat and it removes the unnatural foods that have infiltrated our diet.

However, there is a problem with viewing the carnivore diet as an elimination diet. The Problem Considering the Carnivore Diet as an elimination diet implies that it’s a stepping stone to a different diet. And those next stones give me pause. The Dose-Poison Conundrum “The dose makes the poison.”

This is a famous saying, and for a good reason. There is a lot of truth in it.

Where this gets tricky is that people respond to the same doses with very different effects. Low doses can obviously poison some people and seemingly not affect another. Gluten If someone with Celiac disease eats gluten, the consequences are clear. They have severe GI distress. While someone else may just feel slight intestinal distress. While another may feel no ill effects at all.

A huge issue most of us face is the insidious nature of these toxins. We don’t get immediate and evident feedback. They are “silent toxins.” These silent toxins can gradually increase gut permeability, quietly cells become insulin resistant, oxalates secretly accumulate. And then “suddenly,” seemingly out of nowhere, we end up with an autoimmune disease or diabetes.

Fat Accumulation On average, the American adult gains two pounds per year. After one year it’s not that noticeable. By the end of the decade you are “overweight.” But since culturally we’ve redefined this as the new normal, we just consider this extra weight as “healthy weight”. And then seemingly overnight, you are obese, with diabetes, high blood pressure, and on several prescription medications. (r, r) The Grey Zone There is this grey zone. It blinds us. Because, frankly, it’s hard to see. On one side of the zone, the body handles a toxin just fine – perhaps even a hormetic argument can be made that dealing with some of these toxins makes us stronger, like some argue about the sulforaphane in broccoli. On the other side of the zone is the cumulative impact of daily toxins. They build up. They do damage below our conscious noticing.

Drink a couple beers and you’re fine. Drink a couple beers every night, and the damage done to the liver is not even close to appreciated.

In one sense, someone with Celiac disease who suffers severe digestive distress when eating gluten can consider themselves lucky. They know the dramatic, immediate adverse effect of eating food incongruent with their body. The red flag is loud and clear.

The vast number of people who eat gluten think they are just fine. And since we've come to believe that some digestive distress is completely normal, we don't think twice about the potential gut damage it is doing. Blind to the insidious harm.

Grains make up over half the food consumed in the world. (r) We are exposed to these toxins on a continual basis, meal after meal, day after day, year after year. False Negatives With an elimination diet you gradually add back in other foods to your diet. And if you feel "fine" with them, then it's generally considered "ok" to eat them.

And since you didn't have a reaction to it – it's considered a "negative" response.

But as we just saw, many of these foods are insidious. Eat it once and you are fine. Eat it day after day and you think you're fine. And then "suddenly" the harm finally rears its head as a chronic disease. It was a False Negative. False negatives are the rule, not the exception, with food today. We eat truck loads of sugar, meal after meal, day after day, and we think we feel fine. It's not until we are obese with diabetes

and multiple prescription medication do we finally realize that maybe all the sugar wasn't harmless. Maybe it was a False Negative. False Positives Just the opposite is also a problem. On the opposite side of the spectrum are "false positives."

This means you eat a food, feel terrible, and thus conclude that food is troublesome and to be avoided. But this can be deceiving.

For example, many vegans have turned to the carnivore diet to reverse health problems.

When they start eating a high fat, meat-based diet, they often experience GI distress. It would be easy to conclude that "meat is disagreeable with me."

There can be a painful transition period into the carnivore diet. Yet this would be a "false positive." It seems bad, when really the body is adapting and healing.

It's like after being sedentary for 20 years and then hitting the gym. The pain felt as soreness the next day isn't a negative – it's a false negative – it's a good thing as the body is getting back in shape. A False Negative and False Positive Combined Oxalates are a perfect example that combines a "False Negative" and a "False Positive." You can eat oxalates and not have symptoms (the "False Negative"). But they bioaccumulate in tissues over time.

If allowed to continually build up, these oxalates can form extremely painful crystals resulting in joint pain and kidney stones.

It's not until you stop eating them that the body can finally get rid of the oxalates that have built up. This "oxalate dumping" is often tremendously painful.

It would be easy to conclude, "when I eat oxalates I feel fine (the "False Negative"), but when I removed them I feel terrible" (the "False Positive"). But that false positive is just the body purging a toxin.

But because the causative source is so far removed from the onset of pain, it's hard to see that it was the habitual oxalate consumption that was the culprit. How to use the Carnivore Diet as an Elimination Diet The 3 Carnivore Levels

In "The Ultimate 30-Day Guide to Going Full Carnivore" you'll notice there are 3 "Levels" outlined. With each "Level" more foods get eliminated. By Level 3 – the ultimate elimination diet – just beef (ideally grass fed and finished) and water is eaten.

This 3 Level Framework essentially is an elimination protocol within "Carnivore Approved" foods. For example, Level 1 is the most lenient. You can keep foods like dairy and coffee if

you please. Then in Level 2 it gets a bit more strict, and only meat and water is allowed. Then in Level 3, it's just beef and water. The point of this is that even "carnivore approved" foods like dairy or certain kinds of meat like pork can be problematic.

After such a pure elimination protocol it's easier to identify slight intolerances. But even these aren't immune to the possibility of false positives/negatives.

After Level 3 you may decide to add back in coffee and see how you feel. And you may be just fine, but coffee has plant-toxins, it's a natural insecticide, and it's a food that when people add back in, it's not an occasional thing but typically a daily indulgence. And it's usually not just one cup...

Again the dose is the poison. Coffee is a good example of a risky addition because it makes you feel good (potential false negative) and it's consumed daily. It's not just the occasional treat.

On the flip side, you may be just fine with coffee, you may handle the toxins with ease, and it may not have any negative short term or long term impact.

The problem is you really just can't know. And this is the essential problem with viewing the Carnivore Diet as an Elimination diet. Flexibility vs Relapse The restrictive nature of the Carnivore Diet makes people hesitant to think of it as a long term way of eating. People want to "live life." I get it.

So it's easier to view it as a short term elimination diet to unveil troublesome foods. But there are other ways to implement the Carnivore Diet and "live life."

Some people use the Carnivore Diet as their "baseline" diet – their daily normal – and then allow themselves to deviate on occasions. For some people this allows the flexibility for long term success. For others it a recipe for relapse. If you are like me and one bite turns into eating the whole cake, then often it's just easier not to indulge. I have an "all-in" or "all-out" personality.

For others, one bite can be just one bite. And a flexible approach to the Carnivore Diet gives them the freedom they need to succeed long term. You have to know yourself.

Atkins Diet

I think the built-in "flexibility" is one of the problems people experienced with the popular Atkins Diet. Although Atkins Diet is quite different than the Carnivore Diet, Atkins "Phase 1" can be seen as an elimination diet, and as one progresses, they add in more foods. They add in addictive foods like the sugars in fruits and the carbs in grains. And people fall off the wagon.

It's like taking an alcoholic through rehab, and then saying they can gradually add a few drinks over time. They relapse.

In addition, as one proceeds through Atkins Diet they gradually add in more vegetable oils and more nuts. And even if they don't fall off the wagon, they can still fall prey to "false negatives," as these foods that they add back in become daily staples that can be doing harm without any notice. The Carnivore Diet as an Elimination Diet: Conclusion I think there is danger with viewing the Carnivore Diet as an elimination diet. As is true with any elimination diet it's easy to be deceived by false positives and negatives when adding back in foods. It can easily lead to reverting to previous eating habits and succumbing to sugar and carb addictions.

That said, it can be a very useful tool for some people. As an elimination diet, it can help uncover the worst offenders. In this case, I'd recommend following the directions as laid

out in Level 3 and adding foods back one at a time against a consistent “backdrop” of Level 3.

For those that feel too restricted, instead of viewing the Carnivore Diet as a short term elimination diet, it may be more helpful to adopt a “flexible” approach. While this may lead to relapse for some people, for others it can provide the flexibility for long term success.

Again, it’s essential to “know thyself” as Socrates would say.

The dose is the poison.

Socrates also was well aware of this, as he was sentenced to death by just a touch of poison hemlock – a deadly plant toxin in the carrot family.

So if you choose to use the Carnivore Diet as an elimination protocol just remember when adding back in foods, false positives and negatives can trick you. Doses can be insidious.

These are just a couple things to keep in mind as you tailor your diet to you, and what works in your life, for your goals.

### 30. Titanium implants have been shown to activate the immune system and create chronic inflammation.

a. True

b. False

Bone loss around oral and orthopedic implants: An immunologically based condition

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Abstract Background: Marginal bone resorption has by some been identified as a “disease” whereas in reality it generally represents a condition. Purpose: The present article is a comparison between oral and orthopedic implants, as previously preferred comparisons between oral implants and teeth seem meaningless. Materials and Methods: The article is a narrative review on reasons for marginal bone loss. Results and Conclusions: The pathology of an oral implant is as little related to a tooth as is pathology of a hip arthroplasty to a normally functioning, pristine hip joint. Oral as well as orthopedic implants are recognized as foreign bodies by the immune system and bone is formed, either in contact or distance osteogenesis, to shield off the foreign materials from remaining tissues. A mild immune reaction coupled to a chronic state of inflammation around the implant serve to protect implants from bacterial attacks. Having said this, an

overreaction of the immune system may lead to clinical problems. Marginal bone loss around oral and orthopedic implants is generally not dependent on disease, but represents an immunologically driven rejection mechanism that, if continuous, will threaten implant survival. The immune system may be activated by various combined patient and clinical factors or, if rarely, by microbes. However, the great majority of cases with marginal bone loss represents a temporary immune overreaction only and will not lead to implant failure due to various defense mechanisms.

KEYWORDS adverse immune reaction, bone loss, normal immune reaction, oral implants, orthopedic implants

## 1 | INTRODUCTION

Peri-implantitis, a term derived from the periodontal literature, is often discussed but unclearly defined. Unfortunately, due to ethical restrictions on human experimentation, the majority of studies

relating to peri-implantitis have been performed as ligature studies on experimental animals. By using ligatures of most commonly silk or cotton placed around implants, bone resorption follows that has been concluded to depend on bacteria alone.<sup>1</sup> However, suggestions that the noticed bone resorption solely depends on biofilm formation and

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not the ligature itself<sup>2-4</sup> seem to be incorrect as based on a recent study by C. Dahlin et al. (unpublished data, 2019). C. Dahlin et al. (unpublished data, 2019) placed ligatures around implants placed under sterile conditions in the long bones of rodents and, despite absence of biofilms, bone resorption followed ligature placement anyhow. This study indicates that bacteria may be a secondary phenomenon whereas the primary reaction is an adverse immune reaction to the ligature itself.<sup>1</sup> Success and survival rates for dental implants are very high. There are unknown number of reasons why bone and soft tissues surrounding dental implants become inflamed, swollen, seemingly infected and, in some instances, may lead to implant loss. A scientific published paper search program (Endnotes) was queried in an attempt to determine and understand scientific papers discussing etiologies and treatment for so called peri-implantitis. Two thousand fourteen papers on the cause and various treatment strategies for peri-implantitis were obtained which indicates that this field of research has attracted numerous researchers mainly over the last 15 years. When marginal bone loss around oral implants were scrutinized by some Scandinavian periodontists<sup>5-8</sup> these authors made one assumption, whether directly pointed out or not; they saw implants as being similar to teeth. Therefore, it seemed acceptable to use a term such as peri-implantitis for bone loss around implants because this ailment was regarded as being similar to periodontitis around teeth. When criticized for making pathology of implants that displayed very high clinical success figures, a common response was that periodontitis was a dormant disease, hence this was regarded applicable for peri-implantitis as well. At the time, it was not recognized that teeth and implants had numerous differences; whereas the former represent a natural part of the human body, implants are man-made and, in reality, representing foreign elements eliciting a response from the immune system of the body<sup>9-11</sup> (Figure 1). Recently, osseointegration has been

defined as “a foreign body reaction where interfacial bone is formed as a defense reaction to shield off the implant from the tissues.”<sup>12</sup>

Instead of comparing oral implants and teeth, a more appropriate comparison would be comparing oral and orthopedic implants. Orthopedic implants are foreign bodies too<sup>13-15</sup> and the outcome of a hip arthroplasty is to a major part dependent on immune reactions that, if too strong, will result in implant rejection from the body.<sup>16</sup> Admittedly, there are some differences in the host response to oral implants that display contact osteogenesis<sup>17</sup> and hip arthroplasties that display distance osteogenesis<sup>18</sup>, but these differences are of a much smaller clinical magnitude than previously believed.<sup>19</sup> Therefore, this article is presented as a comparison between oral and hip or knee implants to help us in finding out whether knowledge from the discipline of orthopedics can be applied to the oral implant too.

### 1.1 | Misunderstanding of the past; marginal bone loss solely as an infectious disease concept

The notion that all marginal bone loss around oral implants depends on a biofilm-mediated infection<sup>20</sup> seems to be inspired by findings from research on teeth. However, periodontitis around teeth and marginal bone loss around oral implants have, in fact, little in common.<sup>21-24</sup> Clinical evidence indicates that marginal bone loss around oral implants primarily depends on a complication to treatment due to unsuitable implant designs, poor clinical handling, and patients with different tissue complications<sup>21,25,26</sup> (Figure 2). The presented definition of peri-implantitis by Lindhe and Meyle<sup>27</sup> implying that all bone loss after the implants first year in service combined with inflammation represents a bacterially caused disease is, therefore, unfortunate and leads to very strongly exaggerated figures of so called periimplantitis. The precise mechanism behind marginal bone loss may be quite independent of bacteria,<sup>28</sup> but instead relate to immunological reactions that are intimately associated with the balance between osteoblasts and osteoclasts and in such instances favoring the latter cell type due to reactions to foreign antigens in the implant. Osteoblasts and osteoclasts are bone cells but also a functioning part of the

FIGURE 1 A change from natural dentition to implant-retained rehabilitation. Do the same disease definitions and diagnostic tools still apply?

### 2 ALBREKTSSON ET AL.

immune system.<sup>29,30</sup> Inflammation, incriminated as part of the alleged disease<sup>27</sup> is in reality a normal condition around implants.<sup>9,28</sup> The notion that inflammation without loss of marginal bone represents a pre-stage of disease entitled mucositis<sup>27</sup> is likewise incorrect in the light of an inevitable chronic inflammation around oral implants. Multinucleated giant cells are routinely found in the interface of titanium implants (Figures 3 and 4A,B). In orthopedic surgery, hip implants lose bone too, with so called aseptic loosening being the major reason for hip implant failure. Aseptic loosening was recently reported to depend on innate and adaptive immunological reactions.<sup>16,31,32</sup> However, if the situation with oral implants and marginal bone loss would be similar to what happens in orthopedics rather than with teeth losing bone, a number of basic facts must be analyzed, such as finding out whether the pattern of bone loss is similar between hip arthroplasties and oral implants, whether cp titanium used for the great majority of oral implants actually elicits an immune response which is higher than what is seen in wound healing and whether other materials that are not

osseointegrated such as PEEK and Copper give rise to stronger immune reactions than cp titanium. The presence of aseptic loosening was recently demonstrated in a study on oral implants placed in rats locally injected with titanium particles supporting the notion that immune reactions are behind marginal bone loss.<sup>33</sup> Having said this, antibiotics seem needed in oral implant as well as in joint replacement surgery during the initial phase of implantation to prevent impaired clinical results due to early infections.<sup>16,34</sup> With oral implants, one single preoperative dose of antibiotics was satisfactory to reach a significantly lower failure rate than using no antibiotics at all.<sup>34</sup>

1.2 | The pattern of bone loss and clinical longevity of oral implants and hip replacements  
When oral implants lose marginal bone, the process usually starts in the region of the soft tissues and, if continuous, gradually propagates down toward the apical area of the implant. When hip implants are affected by aseptic loosening, the pattern of bone loss is similar to what is regularly occurring around oral implants; bone loss starts in the proximal region of the hip arthroplasty and, if continuous, eats itself gradually down toward the distal part of the implant<sup>16,35</sup> and the implant fails. Orthopedic implants (Figure 5) have an anchorage

FIGURE 2 Oral implants with marginal bone loss commonly initiated as a complication to treatment and due to immune reactions. Courtesy of Dr C. Ten Bruggenkate

FIGURE 3 A retrieved patient mandibular implant in situ for 6 months. A multinucleated giant cell is seen close to the implant surface

FIGURE 4 A, Multinuclear giant cells (small arrows) forming a continuous layer at a titanium implant surface 7 days after insertion in rabbit bone. Bone formation can be observed (big arrow). FC, fat cells. Electropolishing technique. B, Portion of a multinuclear giant cell at the titanium implant surface (top) 28 days after insertion in the rabbit tibia. The cytoplasm contains numerous mitochondria (m) and a phagocytic vacuole (pv). N, nucleus. Fracture technique

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problem as have oral implants, but the former devices have, in addition, a potential tribologically caused problem; gliding surfaces may elicit numerous wear products not commonly seen around oral implants. These wear products may consist of bone cement<sup>36</sup> of metal fragments<sup>37,38</sup> and of high density polyethylene or plastic particles<sup>39</sup> (Figure 6). The wear phenomenon may result in marginal bone loss due to activation of the immune system.<sup>16,32</sup> Wear particles from cement, metal, and the high-density polyethylene-coated cup result in massive immune reactions that in turn will trigger osteoclasts.<sup>16,40</sup> It is probable that findings from scientific studies of hip replacements may have inspired the work by Wilson et al.<sup>41</sup> on oral implants; these investigators see a combination of implant corrosion and cement leakage to be behind bone loss around oral implants. However, other researchers have added several other factors of importance for marginal bone loss.<sup>24,42</sup> From a clinical longevity point of view, we have clinically documented oral implant materials over 25 years of follow up<sup>43</sup> and in case reports oral implants have survived over 50 years.<sup>24</sup> Cemented Charnley hip arthroplasties were found either functioning or being in situ in 88.2% of cases at 25 years.<sup>44</sup> If a difference in clinical outcome between oral and hip implants, this difference would be limited to young patients who are more prone to failure with hip replacements, presumably due to the risk of overloading of these devices.<sup>45</sup>

### 1.3 | Do sham sites present with smaller immunological reactions than cp titanium, PEEK, and Copper?

Even if metallic implants, in general, today are perceived as being foreign bodies, there has been a dearth of support with respect to whether cp titanium actually presents with a measurable immunological reaction above what is expected from a simple wound healing process. Immune cells are imperative for proper wound healing through the secretion of signaling molecules such as cytokines and growth factors.<sup>46</sup> Therefore, an animal experiment was set up comparing shamoperated sites in one extremity of rabbits with titanium implants placed in the contralateral extremity of the same rabbits. The presence of a titanium implant during bone healing, resulted in a significant activation of the immune system over 10 and 28 days and displayed a type IV (delayed type hypersensitivity) inflammation, likely guiding the host-biomaterial relationship. In addition, bone resorption was suppressed around the titanium implants compared to the sham sites, probably reflecting a stimulus to a strong, protective bone formation around the implant which may be seen as an attempt to isolate the foreign body.<sup>47</sup> In one separate rabbit study, sham-operated sites were used as controls and either titanium, PEEK (used as disk replacements in humans) or copper implants were used as tests in a short-term study spanning over 10 days. Whereas all implants demonstrated a significant elevation of immunological markers, titanium had contact osteogenesis whereas PEEK and Copper implants displayed distance osteogenesis. There was a consistent upregulation of CD4 and downregulation of CD8, indicating a CD4-lymphocyte phenotype antigen-driven reaction around all implants.<sup>48</sup> In yet another rabbit experiment with cp titanium as control, PEEK and Copper were found to display a significantly stronger immunological reaction at 28 days of follow up R. Trindade et al. (unpublished data, 2019). These three animal studies taken together very clearly demonstrate a mild but significant elevation of immunological reactions to cp titanium that presents a significantly stronger reaction compared to operated sham sites but with

FIGURE 5 A hip joint arthroplasty has several materials, such as a metal for anchorage, a ceramic material for gliding surfaces, and a high-density polyethylene in the socket. All these materials may present wear products that may lead to immune reactions

FIGURE 6 Aradiogram of two different types of hip joint replacements. If bone resorption occurs around a hip arthroplasty, the pattern of the bone loss is similar to what may happen around oral implants; bone resorption starts proximally and, if continuous, eats itself gradually toward the apical region

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PEEK and copper presenting even stronger immunological reactions than titanium. Although we are unaware of any detailed studies of the subject, it seems likely that titanium alloys, cobalt-chrome alloys, and stainless steels, frequently used materials in orthopedic surgery, demonstrate similar immunological reactions.<sup>14</sup>

### 1.4 | On bacterial infections and marginal bone loss

Oral implants may be at greater risk for infection than hip arthroplasties; they are placed in the sea of bacteria that exist in the oral cavity. However, the osseointegrated state provides with a defense against bacteria<sup>49,50</sup> and oral implants are usually manufactured from cp titanium with known photocatalytic and bactericidal properties.<sup>51-53</sup> In fact, the defense may start immediately after the placement of the implant, before proper osseointegration has ensued. In a recent study, this bacterial defense was considered to depend on a combination of inflammation and immunological reactions through macrophages.<sup>28</sup> Even

if the present authors strongly oppose theories that bacteria cause all forms of marginal bone loss after the implant's first year in service, we can certainly not exclude that individual cases will display infection as the primary reason for secondary marginal bone loss. Biofilms may result in persistence of implant infections.<sup>54</sup> In case of biofilm-embedded pathogens, those may need much stronger antibiotic concentrations for elimination compared to ordinary bacterial colonies.<sup>55</sup> In experimental cases, it has been demonstrated that biofilms may alter the host innate immune response toward an anti-inflammatory, bactericidal response.<sup>54,56,57</sup> Given the low level of only 0% to 2% secondary failures of oral implants over 10 years or more of follow up,<sup>58</sup> some of those implants may have failed due to infection in combination with adverse immune reactions.<sup>28,59</sup> Numerous other oral implants that display marginal bone loss lack a relation to infection and commonly demonstrate that continued marginal bone loss ceases without treatment at all or ceases after removal of cement residues or other disturbing elements.<sup>28</sup> In orthopedic surgery, it was reported in early clinical long-term observations that there were about 4% of hips failing primarily due to infection.<sup>60</sup> However, the introduction of stringent sterility measures<sup>61</sup> and prophylactic antibiotics reduced the incidence of infection to below 0.2%.<sup>62,63</sup> The placement of hip arthroplasties results in a much greater tissue damage than the placement of oral implants which may result in a greater risk for wound infections with hip replacements. Surgical reaming is conventionally performed until the hip replacement is hammered<sup>64</sup> in stark contrast to the minimal trauma used when placing oral implants according to the principles developed by Brånemark et al.<sup>65</sup> In a total material of 22 170 total hip arthroplasties, 102 hips (0.5%) were revised due to deep infection,<sup>66</sup> but it is possible that the proportion of actually infected hips may be somewhat larger due to shortcomings of revision statistics. In addition, hip replacements revised due to infection, showed an increased rate of infectious complications for the second hip possibly due to surviving bacteria.<sup>67</sup>

Percutaneous orthopedic implants in amputees suffer from greater infection rates (about 20% over 10 years of follow up) than do ordinary hip arthroplasties.<sup>68</sup> As hip replacements of the first generation displayed greater rates of infection than seen today it may be possible to lower these high infections rates in the future. All in all, it seems reasonable to calculate bacteria caused primary bone loss around oral and orthopedic replacement implants to be in the range of about 1% of operated cases, provided adequately trained individuals place clinically documented implant systems. However, even active bacteria act together with the immune system, which may shift the balance between osteogenesis and bone resorption to the domination of the latter. This means that the central issue remains the immune system reaction to the foreign body and in this process bacterial infection is but one of several causative factors. Others are patient smoking, genetic deficiencies, poor clinical handling, implant corrosion, cement residues, and consumption of certain pharmaceutical products<sup>24</sup> that may act together in establishing an aseptic loosening of oral implants through immunological mechanisms precisely as occurs around hip arthroplasties. This means that it seems very likely that the immune system may contribute to success as well as failure of implants.<sup>11</sup>

#### 1.5 | On factors with particular relevance for oral and orthopedic implants

In oral implantology, some clinicians believe they can diagnose implant problems in a similar manner as tooth problems are recorded using particular probes around the devices. Coli et al.<sup>69</sup> pointed out that probing depths of more than 4 mm cannot be seen as a sign of

pathology around oral implants and an increase in probing depths values over time need not automatically be associated with loss of supporting bone around oral implants. In a similar manner, bleeding on probing may only reflect low-grade chronic inflammation and/or release of titanium particles around oral implants instead of being indicative of particular forms of disease. Released titanium particles may act as additional foreign bodies initiating the release of inflammatory mediators associated with bone resorption<sup>70-78</sup> as already described in the case of orthopedic implants.<sup>79</sup> Evaluated sites presented a higher number of titanium particles compared to healthy implants.<sup>80</sup> One important aspect that has not been debated in the literature and that has not yet been properly tested is the fact that probing around oral implants could potentially result in trauma to the periimplant soft tissues leading to inflammation, apical proliferation of the epithelium, and consequent bone loss, particularly as it seems like true probing forces and pressures are higher than suggested values from the literature.<sup>81</sup> There is strong evidence in the literature that the mechanical disruption of the mucosal barrier around an implant should be considered as a connective tissue wound resulting in epithelial proliferation to cover the wound, and in bone resorption to allow a connective tissue barrier of proper dimension to reform to re-establish a “biological width.” Repeated abutment dis/reconnections, with consequent disruption of the peri-implant soft tissue barrier, have been

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shown to cause crestal bone resorption around dental implants in animal studies<sup>82,83</sup> and in short-term and long-term clinical investigations as confirmed in several meta-analysis reports.<sup>84-86</sup> Although this limited crestal bone resorption does not seem to be clinically relevant, this established fact should at least raise the doubt that regular periimplant tissue probing assessments might repeatedly disrupt the soft tissue barrier with consequent serious iatrogenic effects on the stability of the peri-implant tissues in the long term. In an assumed analogy with teeth, plaque has been considered to be one major factor behind problems leading to marginal bone loss at least around experimental oral implants.<sup>87</sup> However, a recent clinical study over a follow up time of 1 to 14 years, reported that plaque accumulation alone did not appear to be associated with bone resorption around oral implants, even if there was evidence of increased soft tissue inflammation.<sup>88</sup> Plaque may in reality form after bone resorption has occurred around oral implants for whatever reason, rather than being the cause of bone resorption. In orthopedic surgery, probing is not used for obvious reasons. Similarities between oral and orthopedic implant problems due to wear products seem obvious, even if the orthopedic implant may see wear not only from the implant material, but also from high-density polyethylene of the cups and from bone cement.<sup>39</sup> Hip implant problems may further ensue from patients with poor bone quality that impairs initial stability due to micro-movements and poor bone healing.<sup>45</sup> Stability is a *conditio sine qua non* for building bone tissue as a foreign body response. If mobility exists, the foreign body response would be characterized by soft tissue capsule formation as described already by Donath et al.<sup>9</sup>

#### 1.6 | Vital statistics related to oral and orthopedic implants

Dental implants are used to replace missing teeth that are lost due to various reasons. Of particular interest is tooth loss caused by an inflammation driven periodontitis that is rarely observed before patient age of 35 to 40 years.<sup>89-91</sup> Patients with such aggressive periodontitis may sometimes lose their teeth completely, despite adequate clinical treatment. Implant treatment to replace missing teeth involves placement of a foreign body

into the host tissue that induces a foreign body immune response that contributes to bone encapsulation of the implants, which become osseointegrated.<sup>9,28</sup> The inflammatory response may vary between patients, and a more severe inflammation that cause problems at the implants within the first year after implant surgery has been observed in some patients.<sup>92</sup> Antoun et al.<sup>93</sup> showed that the prevalence of early inflammation after implant surgery increased from low levels at the age of the patients below 20 years to higher levels in patients in their 50s, where after the risk of inflammation decreased with further increasing age. In other words, the risk pattern of early severe inflammation after implant surgery presented a distribution of a normal curve.<sup>93</sup> This observation suggests a pattern of risk of implant complications that is associated with the level and the intensity of inflammation involved in the process of tooth loss and the age of the patient, and a corresponding level of inflammation after implant placement in the same patients. An inconsistent association between patients with a history of periodontitis and later inflammation at implants has been reported in the literature, indicating both patients with as well as without a higher risk of inflammation at implants after periodontitis problems.<sup>92-96</sup> Thus, patients that lose all teeth early due to severe and treatment resistant periodontitis may in some but not all patients risk greater problems with inflammation and implant failures than others. These patients may further present a higher risk of cardiovascular diseases and early mortality.<sup>4,5,11-13</sup> Similar observations with increased risk of cardiovascular disease and increased mortality in patients between 30 and 50 years of age have been observed in patients with an early onset of osteoarthritis resulting in total hip or knee replacement.<sup>14,15</sup> The mortality ratio decreased with increasing age at the time of hip/knee surgery in these patients, similar to what was observed for edentulous patients provided with dental implants.<sup>11-15</sup> Accordingly, dental as well as orthopedic patients may present a comparable pattern associated to early inflammation driven problems as arthritis or periodontitis, early implant placement in the hip, knee, or oral region, higher risk of cardiovascular diseases and early mortality. A genetic disposition for inflammation susceptibility as observed for patient with cardiovascular diseases cannot be disregarded in these implant patients.<sup>16,17</sup> In essence, in a similar manner as a common etiology between cardiovascular disease and osteoporotic fractures,<sup>95</sup> it seems probable that some patients are particularly sensitive to inflammation which may lead to overreactions to oral or orthopedic implants and to a statistically premature death.

## 2 | DISCUSSION

Implants are foreign bodies that induce a lifelong foreign body response.<sup>9</sup> This inflammation complicates the discussion on “soft tissue health” at the implants and the use of different surrogate endpoints<sup>96</sup> may not be optimal to describe the clinical situation at the implant. A wider approach of the discussion could be to include the immune system and systemic, general, health. The favorable as well as the unfavorable part of the inflammatory response at the dental and orthopedic implant is a complex, multifactorial process, and many variables have been discussed during the years.<sup>97</sup> When a problem is observed at an implant, it could be suggested to reduce strength and numbers of all unfavorable impact factors and to strengthen and include as many favorable response factors as possible. In fact, these principles may indirectly present some criticism to those who analyze the potential outcome of one single factor alone, as single elements one by one would not necessarily display a similar outcome as when the same single element produce a summed

effect with many other factors. Or with other words, the patient does not see overloading alone, but in reality, the patient may be smoking, consuming certain pharmaceutical products, and suffering from other ailments that taken together may threaten the survival of oral and orthopedic implants

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even if each individual factor is below the threshold to cause problems.<sup>42,98</sup> One factor that may lead to marginal bone loss around oral as well as orthopedic implants is bone cement (Figure 7). Dentists use cement as an alternative to screw retaining of mucosa penetrating abutments. It is well-known that this type of cement may accidentally leak out and cause harm to the marginal bone level that gradually is lowered around the implant. The therapy is simple and involves removal of the excess cement. If cement removal is done in time, further bone loss stops immediately and the implant will survive for long, despite some maintained loss of marginal bone.<sup>24</sup> Orthopedic surgeons use cement for anchorage of hip or knee replacements. Components of bone cement are mixed at the surgical theater and when still in a liquid state, the cement will be pressed down into the reamed out marrow cavity of the implant site so that it fills out the space in the trabecular bone in the vicinity. The orthopedic implant is then placed before finished curing of the bone cement, leading to trabecular bone anchorage of the prosthesis. Dental as well as orthopedic cement represents foreign bodies that may threaten the clinical outcome of the implants.<sup>24,36</sup> In oral implantology marginal bone loss has been seen indicative of peri-implantitis, a term derived from the periodontal literature, is often discussed but remains unclearly defined. The term periimplantitis describes a condition (Dorlands Medical Dictionary, 32nd edition), but not necessarily a disease. It is interesting to note that in a recent systematic review, 31 studies were selected for analysis. Inconsistent definitions of peri-implantitis were reported across the studies, indicating a lack of a uniform definition for peri-implantitis.<sup>99,100</sup> The present authors see the diagnosis of marginal bone loss around oral implants to be similar to marginal bone loss around orthopedic implants, that is, a condition but not a disease. Only in the relatively rare cases when primary infection hits oral or orthopedic implants, would it seem motivated to talk about disease. Indeed, the common mechanism behind marginal bone loss around oral or orthopedic implants is of an immunological nature. The immune system reacts to the sum of many different factors with negative impact on the bone and causes marginal bone loss through the impact on immunocompetent cells such as the osteoblast, the osteoclast, and the macrophage. These cells belong to those normally present around implants, but we have previously seen them only as bone related cells without recognizing that they are likewise a part of the immune system. The immune system may change the balance between the osteoblast and the osteoclast in favor of the latter cell type, resulting in resorption of marginal bone. Irrespective if we are considering oral or orthopedic implants, this immunologically determined tissue reaction is the primary reason for loss of anchoring bone tissue, nothing else. Marginal bone loss is seen in similar patterns to oral as well as orthopedic implants. In dentistry, early research on reasons for this bone loss seemed to imply that disease patterns similar to what is seen around teeth occur around implants as well. This is largely misconceived. Instead, the reason for marginal bone loss in oral as well as in orthopedic cases is due to immune reactions. The immune system is always activated by the placement of oral or orthopedic implants because these devices represent foreign bodies. However, as long as we only see a

mild immunological response coupled to mild chronic inflammation, the implant may work well for decades. What we fear is that a series of factors may combine to disturb the steady state of the implants, which in turn may lead to a further activation of the immune system. In case of oral implants, dangerous factors have been identified as patient smoking, having genetic deficiencies or consuming certain pharmaceutical products, adverse clinical handling, implant corrosion, and additional foreign agents such as cement leakage. In case of orthopedic implants, the most incriminated agents have been wear products of metal, plastic, or cement but corrosion or implant overloading may likewise combine to cause marginal bone loss. The mechanism in both cases is activation of the immune system and a shift in the delicate balance between the immunocompetent bone cells, the osteoblast, and the osteoclast resulting in marginal bone resorption. As demonstrated early on in orthopedics but being

FIGURE 7 Cement particles may lead to marginal bone loss around joint replacements as well as around oral implants as in this case

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relevant to oral implants as well is that this process is commonly an aseptic reaction and bacterial actions may be totally absent; aseptic loosening of bone. However, in about 1% of oral and orthopedic implants the bacterial defense may primarily weaken for one reason or the other and similar immunological events follow, but this time started by bacterial attacks. That a plethora of bacteria may be seen by the end of the bone resorbing process around implants is an example of a secondary, not a primary reaction. The foreign body reaction characterized by macrophages and foreign body giant cells was originally defined for soft tissue reactions to biomaterials.<sup>14</sup> However, a similar reaction is seen to titanium in bone during the establishment of osseointegration.<sup>47,48</sup> Bearing in mind that the same primitive mesenchymal stem cells may give rise to different types of connective tissue cells such as fibroblasts and osteoblasts,<sup>101</sup> it seems natural to include bone tissue and osseointegration under a similar umbrella as soft tissue capsules around biomaterials. We, therefore, concur with Donath who described bony demarcation<sup>9</sup> as one mode of tissue defense active to biomaterials such as titanium provided implants are stable in the tissues. This bony demarcation is the same as osseointegration that presents most successful clinical long-term results in the great majority of treated patients.<sup>58</sup>

Conclusions

1. Oral and orthopedic implants represent foreign bodies that activate the immune system
2. Oral and orthopedic implants function very well at least over about 25 years of follow up
3. The pathology of an oral implant is as little related to a tooth as is pathology of a hip arthroplasty to a normally functioning, pristine hip joint
4. An over-reaction of the immune system is the major reason for marginal bone loss and failure of oral and orthopedic implants

### 31. Which statement is incorrect?

- Fluoride ions from mouthwash, toothpaste, drinking water, and food could potentially cause corrosion on titanium implant surfaces.
- Titanium particles are released from an implant surface after it is cleaned with ultrasonic scaling instruments
- Both A and B are incorrect.
- Both A and B are correct.

Increased Levels of Dissolved Titanium are Associated With PeriImplantitis – A Case-Control Study Luciana M. Safiotti, DDS, MS\*, Georgios A. Kotsakis, DDS, MS\*, Alex E. Pozhitkov, PhD†, Whasun O. Chung, PhD†, Diane M. Daubert, MS\* \*Department of Periodontics, University of Washington, Seattle, WA. †Department of Oral Health Sciences, University of Washington, Seattle, WA. Background. Peri-implantitis represents a disruption of the biocompatible interface between the titanium (Ti) dioxide layer of the implant surface and the peri-implant tissues. Increasing preclinical data suggest that the peri-implantitis microbiota not only triggers an inflammatory immune response but also causes electrochemical alterations of the Ti surfaces, i.e. corrosion, that aggravate this inflammatory response. Thus, we hypothesized that there is an association between the dissolution of titanium from dental implants, which suggests corrosion, and periimplantitis in humans. The objective of this study was to compare the levels of dissolved titanium in submucosal plaque collected from healthy implants and implants with peri-implantitis. Methods. Submucosal plaque from N=20 implants with peri-implantitis and N=20 healthy implants was collected with sterile curettes (N=30 participants). Levels of titanium were quantified using inductively coupled plasma mass spectrometry (ICP-MS) and normalized for mass of bacterial DNA per sample to exclude confounding by varying amounts of plaque per site. Statistical analysis was performed utilizing Generalized Estimated Equations (GEE) to adjust for clustering of implants per subject. Results. Implants with peri-implantitis harbored significantly higher mean levels of titanium ( $0.85 \pm 2.47$ ) versus healthy implants ( $0.07 \pm 0.19$ ) after adjusting for amount of plaque collected per site (p-value=0.033). Conclusions. Greater levels of dissolved titanium were detected in submucosal plaque around implants with peri-implantitis when compared to healthy implants, indicating an association between Ti dissolution and periimplantitis. Factors triggering titanium dissolution as well as the role of titanium corrosion in the peri-implant inflammatory process warrant further investigation.

KEY WORDS: biofilms, corrosion, dental implants, peri-implantitis, titanium. Titanium dental implants are widely utilized to replace missing teeth owing to their ability to form a direct structural and functional connection with host bone.<sup>1,2</sup> Branemark coined the term “Osseointegration” in 1977 to describe this biologic phenomenon and paved the way for contemporary implant practice.<sup>3</sup> Titanium, a metal with excellent biocompatibility, is the most frequently utilized biomaterial for the construction of dental implants primarily because of the formation of a titanium dioxide (TiO<sub>2</sub>) layer that yields high resistance to corrosion.<sup>4,5</sup> Nonetheless, despite the high resistance to corrosion and biocompatibility properties attributable to titanium, corrosion of dental implants can still happen under certain circumstances in the oral environment. Corrosion processes lead to physicochemical alterations on the implant surface that include disruption of the TiO<sub>2</sub>

layer and facilitate titanium dissolution.<sup>6</sup> A multitude of factors may lead to corrosion. Corrosion-triggering factors include local acidification due to inflammation of the peri-implant tissues which may modify the corrosion resistance of titanium,<sup>7</sup> or promotion of an acidic environment by bacteria such as *Streptococcus mutans*, due to the release of lactic acid, which promotes a decreased corrosion resistance of titanium in vitro.<sup>8</sup> Interestingly, *S. mutans* has been found in higher levels around implants with peri-implantitis when compared to healthy

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implants and to teeth with periodontitis.<sup>9</sup> This finding implies that the specific bacterial niche associated with peri-implant microenvironments might have a role in addition to the interaction with host immune responses that may also contribute to peri-implantitis. In addition to bacteria, aggressive chemical stimuli such as acidic fluoride solutions have been associated with corrosion.<sup>10, 11</sup> Furthermore, the electrical conductivity of titanium in the presence of oral bacteria has been shown to provide a closed circuit that may enhance the bio-corrosive process.<sup>12</sup> Irrespective of the etiologic factor for the initiation of titanium corrosion, the resulting corroded surfaces of dental implants may offer amenable niches for the colonization by bacteria. *Porphyromonas gingivalis* has shown increased attachment to corroded titanium disks when compared to non-corroded titanium disks indicating that a corroded surface may promote increased bacterial colonization.<sup>13</sup> In addition, lipopolysaccharides from gram-negative bacterial wall have demonstrated a high affinity to titanium, while also promoting dissolution of the TiO<sub>2</sub> layer in pH 4-7 which is found in the oral environment.<sup>7</sup> Thus, peri-implant bacteria and chemical factors work in synergy having corrosion as the common denominator to their deleterious effects on peri-implant health. Titanium particles and ions leaked from dental implants are not bioinert; corrosion products possess an immunogenic potential acting as secondary stimuli for the inflammatory process in peri-implantitis and may enhance bone resorption.<sup>14</sup> Mice immune cells stimulated by lipopolysaccharide (LPS) in the presence of Ti ions show increased release of pro-inflammatory cytokines involved in bone resorption.<sup>15</sup> In addition, titanium particles have been shown to induce the release of TNF- $\alpha$  when phagocytized by macrophages,<sup>16</sup> increase the release of IL-1 $\beta$  by macrophages,<sup>14</sup> and induce the secretion of RANKL upon entering T-cells<sup>17</sup> stimulating bone resorption, indicating corrosion as a potential etiologic factor in the pathogenesis of periimplantitis. In vivo, titanium particles have been found in soft and hard tissue biopsies retrieved from sites with peri-implantitis,<sup>18,19</sup> and in epithelial cells surrounding implants with and without peri-implantitis.<sup>20</sup> However, the question remains to whether an association exists between the presence of titanium dissolution and peri-implantitis. Therefore, the objective of this study was to compare the levels of titanium dissolution in submucosal plaque samples collected from healthy implants and implants with peri-implantitis. We hypothesized that submucosal plaque around implants with peri-implantitis will harbor higher levels of titanium particles when compared to healthy implants.

MATERIALS AND METHODS

**Setting** This case-control clinical study took place at the Graduate Periodontics clinic, School of Dentistry, University of Washington. Recruitment and data collection started in May 2015 and ended in February 2016.

**Participants** Thirty patients of the Graduate Periodontics clinic at the University of Washington were recruited for the study. All patients signed and received a copy of an informed consent approved by the Institutional Review Board at the University of Washington.

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### 3

**Inclusion criteria** consisted of one healthy implant and/or one implant with peri-implantitis, implants loaded with restorative components for at least 2 years, and presence of a baseline and/or current radiograph of the implant restored. Various implant brands were included in the study. **Exclusion criteria** consisted of history of systemic or local antibiotics in the last three months.

**Peri-implantitis Assessment** The clinical parameters recorded for each implant included probing depths at six sites, bleeding on probing and/or suppuration, Plaque Index score,<sup>21</sup> Gingival Index score,<sup>21</sup> and number of years of implant in function after loading. Peri-implantitis diagnosis was ascertained based on clinical and radiologic criteria according to existing recommendations.<sup>22, 23</sup> In detail, a diagnosis of peri-implantitis was rendered for implants having probing depths  $\geq 5$  mm, bleeding on probing and/or suppuration, and bone loss  $\geq 2$  mm (Figure 1). In the absence of previous radiographic records, a vertical distance of 2 mm from the expected marginal bone level following postsurgical remodeling was used to depict radiographic bone loss.<sup>23</sup> Implants with probing depths  $< 5$  mm, no bleeding on probing, no suppuration, and no evidence of radiographic bone loss were considered as being healthy (Figure 2). A consensus among three independent reviewers (LS, GK, DD) was required for assigning diagnoses. Additional variables recorded included subject age, gender, smoking and diabetes status.

**Assessment of Titanium Content in Submucosal Plaque Samples** Prior to sample collection, supramucosal plaque was removed. Submucosal plaque samples were collected from the deepest probing sites at each dental implant with sterile mini-five 1-2 Gracey curettes with the curette edge facing away from the implant surface. Care was taken not to contact or scratch the implant surface with the curette. Submucosal plaque samples were stored in 500  $\mu$ l of sterile water in screw-cap tubes and frozen at  $-80^{\circ}\text{C}$  for future processing. A 350  $\mu$ l aliquot of the collected sample was utilized for quantitation of titanium by Inductively Coupled Plasma Mass Spectrometry (ICP-MS $\ddagger$ ). A 150  $\mu$ l aliquot was used for DNA isolation and DNA quantification to verify the amount of plaque in each sample.

**Inductively Coupled Plasma Mass Spectrometry** The samples were processed as previously described.<sup>12</sup> They were transferred to digestion vessels (50 mL polypropylene centrifuge tubes) with four 1 ml rinses of digestion solution (50:50 (V/V) concentrated nitric acid trace-metal grade $\S$ : deionized (DI) water $\parallel$  with a trace amount of hydrofluoric acid $\nabla$  and 10 ppm terbium $\nabla$  as recovery standard. Each sample was brought to 5 ml with the digestion solution. Open vessel microwave $\#$  digestion was used (power 800W, 100%, ramp

15 min to 100°C, hold for 45 min). After the digestion, samples were brought to 25 ml with DI water. Analysis for Ti was conducted by ICP-MS with a detection limit of 0.5 ng. DNA Isolation and Quantification DNA from the plaque aliquots was isolated using a styrene divinylbenzene copolymer containing paired iminodiacetate ions,\*\* which act as chelating groups in binding polyvalent metal ions. A 150 µl aliquot of the plaque sample was placed into a tube containing 10 mg of the copolymer followed by addition of 50 µl of 120 mM Tris HCl pH 8.0 followed by addition of 10 µl of 10 mg/mL proteinase K. The mix was incubated at 55°C for 30 min followed by vortexing and incubation in a boiling-water bath for 8 min. Following the boiling water bath, the tubes were centrifuged at 10,000–15,000×g for 3 min and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube.<sup>12</sup> The amount of DNA per sample was quantified with a device++ set for analysis of DNA using 2 µl of each sample for quantification of DNA present in the sample. The quantity of DNA per µl was utilized to calculate the amount of titanium present adjusted to the amount of bacterial DNA.

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mg/mL proteinase K. The mix was incubated at 55°C for 30 min followed by vortexing and incubation in a boiling-water bath for 8 min. Following the boiling water bath, the tubes were centrifuged at 10,000–15,000×g for 3 min and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube.<sup>12</sup> The amount of DNA per sample was quantified with a device++ set for analysis of DNA using 2 µl of each sample for quantification of DNA present in the sample. The quantity of DNA per µl was utilized to calculate the amount of titanium present adjusted to the amount of bacterial DNA.

Statistical Analysis Sample size estimation was performed based on previously published results by Olmedo et al. 20 who assessed levels of titanium particles by ICP-MS in pooled samples from epithelial cells around implants with peri-implantitis when compared to healthy implants. Based on the effect size they observed, twenty implants per group (N=20) were deemed adequate in the present study to yield 90% power to detect a significant difference between groups using a test of means at alpha=0.05 and accounting for 30% attrition due to sampling / assay errors. Summary statistics were reported for all variables and non-parametric tests were used for continuous variables and Pearson's chi-square tests were used for categorical data. To eliminate confounding by higher amount of submucosal plaque around implants with peri-implantitis compared to healthy implants, the titanium levels were normalized to the amount of plaque/site by using the quantity of bacterial DNA present in an aliquot of the sample prior to statistical comparisons. Generalized Estimating Equations were used for comparison of log-transformed adjusted titanium levels between groups to adjust for clustering of implants within the same subject. Statistical significance was set at p-value < 0.05.

## RESULTS

Participants Thirty patients (11 females, 19 males) providing forty implants were recruited; twenty healthy implants and twenty implants with peri-implantitis. The mean patient age was  $70.25 \pm 7.98$  in the healthy group and  $67.1 \pm 9.37$  in the peri-implantitis group. Fifty percent of the implants in the healthy group were placed in females, while eighty percent of the implants in the peri-implantitis group were placed in females. The number of years of implants in function was  $8.12 \pm 4.33$  in the healthy group and  $7.95 \pm 4.59$  in the peri-implantitis group. Number of smokers and persons with diabetes did not significantly differ between groups (p-values 0.69 and 0.65, respectively). The probing depths, Plaque Index and the Gingival Index were significantly greater in the periimplantitis group (p-values 0.001, 0.04 and 0.001, respectively) (Table 1). Various

implant systems were included in this study but no comparisons among brands have been conducted because we were not powered to perform such comparisons.

Levels of Titanium Amounts of plaque collected were significantly higher in the peri-implantitis group ( $48.73 \pm 67.16$ ) than in the healthy group ( $23.87 \pm 14.06$ ) ( $p$ -value=0.01) (Figure 3), thus titanium levels per group were adjusted for ng/ $\mu$ l of plaque collected as discussed above. In implant level analysis, mean titanium levels were significantly higher in diseased ( $0.85 \pm 2.47$ ) versus healthy

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implants ( $0.07 \pm 0.19$ ) after adjusting for amount of plaque collected per site ( $p$ -value=0.03) (Figure 4). In multilevel analysis with GEE, results showed significantly greater titanium levels at the peri-implantitis group as compared to the healthy group ( $p$ -value=0.03), which remained consistent in multivariable GEE analysis when further adjusting for smoking status (adjusted  $p$ -value=0.82) and years of implant in function (adjusted  $p$ -value=0.76).

**DISCUSSION** To our knowledge, this is the first study to find a statistically significant higher quantity of titanium dissolution in submucosal plaque around implants with peri-implantitis when compared to healthy implants. Titanium dissolution suggests corrosion of the dental implant surface. This significant association between titanium dissolution and peri-implantitis implicates corrosion as an important factor in peri-implantitis research. Previous studies have shown the presence of titanium particles in peri-implant tissues, however no statistical evidence of an association between dissolved Ti and peri-implantitis has been reported. Ti particles have been found in epithelial cells exfoliated from peri-implant tissues,<sup>20</sup> foreign bodies consisting of titanium and cement have been found in soft tissue of implants with peri-implantitis,<sup>19</sup> and Ti elements have been found in bone and soft tissue biopsies retrieved from sites with peri-implantitis.<sup>18</sup> Olmedo et al.<sup>20</sup> introduced exfoliative cytology to assess the presence of titanium particles in cells exfoliated from the peri-implant mucosa. Using pooled samples, the authors found a higher concentration of titanium in the peri-implantitis group when compared to healthy implants. The present results are well aligned with the findings of Olmedo et al. and elucidate the role of periimplant plaque as an intermediary pool of titanium particles that may subsequently be transferred into the host tissue. Our study utilized submucosal plaque as a non-invasive and easily accessible media for collection and subsequent quantification of relative amounts of dissolved titanium. Curettes were used for sampling since they are considered superior to paper strips as a means of collecting bacterial DNA for quantitative analysis.<sup>24</sup> This approach was found to be robust for the analyses performed in the study since; 1) a significant higher quantity of submucosal plaque was collected from sites with peri-implantitis as expected due to the presence of deeper probing depths and disease activity at those sites, and; 2) titanium levels in the healthy group were very close to zero confirming that the use of the curettes did not lead to interfere with the implant surface. Previous work has recorded the immunogenic effect of titanium corrosion products' participation in amplifying the host inflammatory response and enhancing bone loss in periimplantitis lesions by means of an increased release of pro-inflammatory cytokines,<sup>15</sup>

TNF- $\alpha$ , IL-1 $\beta$ , IL-17 and RANKL by host cells in the presence of titanium products. Nonetheless, the present work unveiled a previously unexplored association between bacterial plaque, dissolved titanium released into the peri-implant tissues and peri-implantitis. A pathogenic cycle can be speculated originating from the interaction between titanium and bacterial plaque that leads to definitive alterations on the titanium surface, while bacterial attachment and growth is propagated on the corroded surface. Pathogens such as *S. mutans* have the ability to cause corrosion due to the creation of an acidogenic environment, and LPS and pathogens such as *P. gingivalis* have a high affinity for adsorption to those corroded titanium surfaces. Titanium is not bacteriostatic for most bacterial species,<sup>25</sup> therefore providing a suitable environment for colonization. Furthermore, it has been found that the expression of TLR-4 (an LPS receptor) in gingival epithelium exposed to titanium ions was elevated when compared to that of titanium  
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free samples, indicating that titanium ions may increase the sensitivity of gingival epithelial cells to microorganisms in the oral environment.<sup>26</sup> The interaction and synergistic effects between the biofilm and the dissolved titanium deserve detailed investigation in an attempt to understand its effects in the pathogenesis of peri-implantitis. Lastly, the implication of corrosion in peri-implantitis raises awareness of the distinct differences between peri-implantitis and periodontitis. Treatments currently aimed at resolving or controlling peri-implantitis include non-surgical and surgical therapy utilizing approaches identical to those targeted to the treatment of periodontitis around teeth. However, the evident association between peri-implantitis and dissolved titanium raises the importance of the development of therapies that aim to address not only the control of biofilm, but also respect the titanium surface material properties. Agents that may be protective for teeth, such as fluoride, can be detrimental to titanium leading to corrosion.<sup>10, 27, 28</sup> Titanium from dental implants can be exposed to fluoride ions from mouthrinses, toothpastes, drinking water, or food,<sup>29</sup> and the use of fluoride could be a potential confounding factor to be considered in future confirmatory studies assessing the implication of titanium corrosion and peri-implantitis. The importance of developing implant-specific treatment protocols that factor in titanium cytocompatibility has only recently been realized<sup>30</sup> and the present findings corroborate the need to consider titanium material properties and its biocompatibility for establishing peri-implantitis treatment strategies that will bode for long-term success.

**CONCLUSION** Significantly increased levels of dissolved titanium were detected in submucosal plaque of implants with peri-implantitis when compared to healthy implants, thus indicating an association between titanium dissolution and peri-implantitis. The factors triggering titanium dissolution as well as the role of titanium corrosion products in the peri-implant inflammatory process warrant further investigation.

Scaling of titanium implants entrains inflammation-induced osteolysis Michal Eger<sup>1</sup>, Nir Sterer<sup>2</sup>, Tamar Liron<sup>1</sup>, David Kohavi<sup>2</sup> & Yankel Gabet<sup>1</sup>

With millions of new dental and orthopedic implants inserted annually, periprosthetic osteolysis becomes a major concern. In dentistry, peri-implantitis management includes cleaning using ultrasonic scaling. We examined whether ultrasonic scaling releases titanium particles and induces inflammation and osteolysis. Titanium discs with machined, sandblasted/acid-etched and sandblasted surfaces were subjected to ultrasonic scaling and we physically and chemically characterized the released particles. These particles induced a severe inflammatory response in macrophages and stimulated osteoclastogenesis. The number of released particles and their chemical composition and nanotopography had a significant effect on the inflammatory response. Sandblasted surfaces released the highest number of particles with the greatest nanoroughness properties. Particles from sandblasted/acid-etched discs induced a milder inflammatory response than those from sandblasted discs but a stronger inflammatory response than those from machined discs. Titanium particles were then embedded in fibrin membranes placed on mouse calvariae for 5 weeks. Using micro-CT, we observed that particles from sandblasted discs induced more osteolysis than those from sandblasted/ acid-etched discs. In summary, ultrasonic scaling of titanium implants releases particles in a surface type-dependent manner and may aggravate peri-implantitis. Future studies should assess whether surface roughening affects the extent of released wear particles and aseptic loosening of orthopedic implants. The number of dental and orthopedic implant patients is increasing with an estimated 5 million new dental implants ([www.persistencemarketresearch.com](http://www.persistencemarketresearch.com)) and similar numbers of orthopedic prostheses each year worldwide. In dentistry, peri-implantitis is a recent but already major clinical concern and the main cause of long-term implant failure<sup>1–3</sup>. Peri-implantitis is triggered by specific oral bacteria and consists of inflammation that leads to bone resorption (osteolysis) around dental implants<sup>4</sup>. Once the peri-implant process starts, it can rarely be controlled and often results in implant loss<sup>5</sup>. Although there is no well-established treatment protocol for peri-implantitis, treatment usually begins with conservative attempts such as initiating mechanical cleaning of the surrounding biofilm using ultrasonic scaling and the use of antiseptic solutions and local and systemic antibiotic administration<sup>2,3</sup>. When these options fail to restrain disease progression, the implant is surgically removed. The bond between a living bone and the implant surface is believed to be an important factor in clinical implant success. The factors contributing to osseointegration include implant composition, geometry and surface energy and texture<sup>6,7</sup>. Dental implant surfaces have been continuously modified, ranging from relatively smooth, machined (a.k.a. ‘turned’) surfaces to roughened surfaces. The roughening of implant surfaces is accomplished by physical procedures that involve blasting by various substances and/or acid etching<sup>6,8,9</sup>. Currently, virtually all commercially available implants undergo surface roughening. The majority of published reports show that there are benefits to using micro-roughening versus machined titanium (Ti) surfaces during the early healing stages of implant integration (up to three months)<sup>6,7</sup>. However, after three months, the use of rough implants presents no clear advantages<sup>1</sup>. Moreover, it is more challenging to prevent and manage peri-implantitis when using roughened implants instead of machined implants<sup>1</sup>. Although Ti is considered to be a biocompatible material, long-term joint loosening after orthopedic joint replacement has been associated with aseptic inflammation. This inflammation is thought to be caused by

the Ti particles that are released from the implant surface into the surrounding micro-environment, where they are

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phagocytosed by circulating monocytes and macrophages<sup>10,11</sup>. Macrophages engulf Ti particles causing them to secrete inflammatory cytokines, such as IL1 $\beta$ , IL6 and TNF $\alpha$ . These secreted cytokines are strong inducers of osteoclastogenesis and bone resorption<sup>12</sup>. Here, we hypothesized that dental implant scaling, a procedure that is aimed at preventing peri-implantitis, may actually cause and aggravate the progression of the disease. We therefore examined the release of Ti particles from several surface-treated Ti discs following ultrasonic scaling and determined the effects of these particles on inflammation and associated bone loss in vitro and in vivo. In these experiments, we aimed to characterize the factors that contribute to Ti particle-induced inflammation. Results Titanium Disc and Particle Characterization. Titanium Discs. To examine the topographic differences between Ti surfaces on machined (M), sand-blasted (SB) or sand-blasted and acid-etched (SLA) discs, we analyzed their nanoroughness using Atomic Force Microscopy (AFM, Table 1). Nanoroughness was characterized according to Z-range, Rq, Ra, R-max and surface area differentiation. The SB discs were found to have the greatest roughness out of all examined parameters, except surface area differentiation. SLA showed the second greatest values for all parameters, except surface area differentiation, for which it had the greatest value. The M surfaces displayed the lowest values for all roughness-related parameters. The XPS test for chemical composition revealed that SLA and SB surfaces were more contaminated than M surfaces by elements that were not related to the Ti alloy, such as Si, P, Ca and Zn (Table 2). Half of the surface of each disc was scaled using a dental ultrasonic scaler, and the effect of scaling on each Ti surface was assessed using Scanning Electron Microscopy (SEM, Fig. 1a,b). As expected, scaling resulted in a smoother topography on the rough surfaces (SLA and SB). In contrast, the machined surface showed a relatively more irregular surface after scaling. Qualitatively, all 3 surface types, which were characterized by their very distinct topographies before scaling was performed, displayed a similar pattern after scaling.

M SLA SB Z range (nm) 670.00 4049.17 4414.67 Rq (nm) 100.15 680.83 763.33 Ra (nm) 80.40 558.33 617.83 R max (nm) 661.33 3963.50 4421.33 Surface area differentiation (ratio) 0.03 0.34 0.27 Table 1. Nanoroughness characterization of M, SLA and SB titanium discs, measured using AFM.

Titanium Discs (XPS)

Titanium Particles (EDS)

US Tip (EDS) M SLA SB M SLA SB — C 33.04 26.65 25.00 19.65 28.622 25.74 22.49 O 47.25 49.96 48.82 15.17 23.30 28.63 ND Ti 13.02 15.94 9.47 56.82 40.25 33.19 ND Al 2.71 2.91 13.22 4.89 3.28 4.9 0.55 V 0.25 0.33 0.12 2.77 2.01 1.70 ND N 2.82 2.95 1.18 ND ND ND ND S 0.55 0.62 0.39 ND ND ND ND Cl 0.16 ND 0.06 ND ND ND ND ND Pb 0.21 0.01 ND ND ND ND

ND Si ND 0.16 0.75 ND ND ND 0.17 P ND 0.09 ND ND ND ND ND Ca ND 0.04 0.22 ND ND ND 0.05 Zn ND 0.33 0.08 ND ND ND ND Cr ND ND ND 0.05 0.35 0.04 13.45 Fe ND ND ND 0.61 2.13 0.44 64.24 Cu ND ND ND 0.04 0.048 0.12 1.78 Ni ND ND ND ND ND ND 1.63 F ND ND ND ND ND 2.36 Table 2. Chemical characterization of M, SLA and SB titanium discs and particles. The chemical profiles of sterilized titanium discs were analyzed using XPS (elements determined according to the Mendeleev periodic table). The titanium particles that were released by US scaling and the US tip were analyzed using EDS.

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Figure 1. Topographic changes and titanium particles produced by US scaling. Three different titanium surfaces, including M, SLA, and SB, were treated with US scaling on half of the disc area. (a) The border was examined using SEM at  $\times 300$  magnification. (b) The areas on both sides of the midline were examined at  $\times 1500$  magnification. In each panel, the left side represents the original disc surface, while the right side shows the disc after treatment. (c) Titanium particles released during this process were visualized using SEM to estimate their micro-roughness, (d) the number of particles and (e) the size distribution of the particles, which were evaluated using an automated cell counter. The size distribution is shown as a percentage of the total number of particles that originated from each surface type. (f–h) Nanoroughness of particles was analyzed using atomic force microscopy (AFM). (f) Multiple line profiles were obtained for each particle type. (g) Slope, the fold-increase in linear distance and the average maximal distance between the highest and deepest points along 500 nm were calculated to determine particle nanoroughness. (h) Representative line profiles are shown for the different surfaces.

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Titanium Particles. We further assessed the differences between the Ti particles that were released from each surface. The numbers and size distributions of the particles that were released by scaling were evaluated using SEM and quantified using an automated Micro-Counter. The number of particles per surface area varied greatly among surfaces and was 48.5, 89.8 and 121.3 thousand particles/mm<sup>2</sup> for the M, SLA, and SB discs, respectively (Fig. 1d). No significant difference was found in the sizes of the particles ( $7.57 \pm 1.43$ ,  $7.57 \pm 2.75$  and  $8.37 \pm 2.94 \mu\text{m}$  for M, SLA and SB, respectively) or in their size distributions. The majority of the particles were within the 6- to 8- $\mu\text{m}$  range (Fig. 1e). High-resolution SEM images (10,000 $\times$  magnification) of the released particles suggested that there were differences in particle roughness (Fig. 1c). To confirm this finding, particle nanoroughness was examined using AFM-based line profilometry (Fig. 1f). Isolated particles were characterized for profile line steepness, linear distance differentiation ('pseudo-Ra') and peak-to-valley mean height ('pseudo-Rz'). Steepness and linear distance differentiation were significantly higher for SB-derived particles, whereas the peak-to-valley mean height was significantly lower for SLA-derived particles (Fig. 1g). Energy-Dispersive X-ray spectroscopy (EDS) was used to evaluate the chemical composition of the particles (Table 2). Particles originating from all three Ti surfaces were contaminated with Cr, Fe, and Cu metal elements that were not found in the discs. To determine the origin of these metals, we analyzed the chemical composition of the ultrasonic tip that was used for disc scaling. All three metals, among others, were found. We therefore assumed that the contamination by Cr, Fe, and Cu originated from the scaler tip (Table 2). Our data indicate

that the numbers, nanoroughnesses and chemical profiles, but not the average sizes of the released particles, differed among the 3 surface types.

**Inflammatory Response and Osteoclastogenesis Induced by Titanium Particles.** Ti particles have been repeatedly reported to induce an inflammatory response in vivo in the tissue surrounding implants and in vitro<sup>11,13,14</sup>. However, the pro-inflammatory response to the particles that originated from the ultrasonic scaling of dental implants has not yet been studied. Primary bone marrow-derived macrophages (BMDMs) were cultured for 24 hours with the Ti particles that were released by ultrasonic scaling of the SLA surface, which is the most common surface treatment that is used in commercially available dental implants. This setting is clinically relevant because it mimics the release of particles that occurs during routine cleaning around dental implant. Bacterial lipopolysaccharide (LPS) was added to a parallel set of cultures as a positive control<sup>15</sup> and to Ti particles to assess additive/synergistic effects. The gene expression profiles of pro-inflammatory cytokines (IL1 $\beta$ , IL6, TNF $\alpha$ ) indicated that the Ti particles induced a greater inflammatory response than 0.01  $\mu$ g/ml LPS (a 40–70-fold increase,  $p < 0.001$ ). The response to both LPS and Ti particles appeared to be additive rather than synergistic (Fig. 2). Because RNA expression does not always reflect secretion of these cytokines, we also analyzed protein levels of IL1 $\beta$ , IL6, TNF $\alpha$  in the supernatant of the macrophage cultures. Our multiplex analysis revealed a trend very similar to our RT-qPCR analysis with cytokines levels being the highest in the macrophages cultured with Ti particles (Fig. S1). To test whether the presence of Ti particles affects cell viability, we performed an apoptosis assay. We found that neither LPS nor Ti affected macrophage survival (Fig. S2). To test the effect of titanium particles on osteoclast differentiation, we cultured pre-osteoclasts with LPS, SLA Ti particles or diluent alone in an osteoclastogenic medium. Notably, in the absence of RANKL, none of these conditions generated tartrate-resistant acid phosphatase (TRAP)<sup>+</sup>, multinucleated osteoclasts (Fig. S3). However, in the presence of RANKL, we were able to measure the effect of Ti particles on osteoclastogenesis. Similar to the inflammatory response, the presence of SLA particles significantly stimulated the size and total area of TRAP<sup>+</sup> cells to levels above those observed in both the control and LPS-treated cultures (Fig. 3,  $p < 0.005$ ). Based on our observation that scaling the different surfaces resulted in differences in the number of particles, we next assessed the effect of the number of particles on the inflammatory response. In these experiments, we cultured BMDMs with increasing numbers of particles that originated from SLA discs. Increasing the number of particles exponentially increased the inflammatory response, as indicated by IL $\beta$ , IL6, and TNF $\alpha$  expression levels (Fig. 4a). A regression analysis revealed that the inflammatory response (y) that was induced by the density of particles (x) could be predicted using the following formula:

$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4 + \beta_5 x^5 + \beta_6 x^6 + \beta_7 x^7 + \beta_8 x^8 + \beta_9 x^9$

with goodness of fit of  $R^2 = 0.97$  for IL1,  $R^2 = 0.93$  for IL6, and  $R^2 = 0.89$  for TNF $\alpha$ .

To determine whether the extent of the inflammatory response is dependent upon the original implant surface, BMDMs were cultured with 10% of the particles that were released by scaling one disc (28.27 mm<sup>2</sup> area) with each surface type, which corresponded to 137 thousand M disc-produced particles, 254 thousand SLA disc-produced particles and 343 thousand SB disc-produced particles. The surface of the discs is equivalent to the exposed area in a 3.75-mm diameter implant following ~2 mm of vertical crestal bone resorption (Fig. 5). Each type of particle was placed in the well of a 6-well

plate (9.6 cm<sup>2</sup> area). An analysis of cytokine expression indicated that the particles originating from SB discs induced the most severe inflammatory response, whereas particles from machined surfaces induced the mildest response (Fig. 4b). As mentioned above, there was no difference in the average size of the particles released from different implant types, but they did display distinct nanotopographies and chemical profiles. To assess whether the extent of the inflammatory response is dependent not only upon the number but also the type of original implant surface, BMDMs were cultured in 6-well plates with an equal number of particles, 1293 particles/mm<sup>2</sup>, from each surface type (M, SLA, or SB). This approach was aimed at nullifying the effect of particle number. The analysis of cytokine expression indicated that particles originating from SB discs induced the most severe inflammatory response, whereas the mildest response was observed in cells exposed to particles from machined surfaces (Fig. 4c). Overall,

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nanoroughness and/or chemical composition (which is dependent on the implant surface of origin) and particle number appear to have similar effects on the expression of pro-inflammatory cytokines in macrophages.

**Titanium Particles Stimulate Inflammatory-Induced Osteolysis in vivo.** The effect of titanium particles on bone resorption was examined using a mouse calvaria model. Here we used 1.2 million particles for 'SLA' membranes and 1.8 million particles for 'SB' membranes. This number corresponds to the total number of particles released by US scaling of half a disc with SLA and SB surface respectively. In line with our in vitro results, 3D images reconstructed from the  $\mu$  CT data clearly showed that the surface of the parietal bone was eroded in the Ti particle groups (Fig. 6a). Pit resorption volume (PRV) and the pit resorption volume/tissue volume (PRV/TV) ratio in the ROI were measured.

Quantitatively, particle-induced osteolysis was significantly higher in the titanium groups than in the sham controls. Moreover, bone loss was dramatically more severe in the SB group than in the SLA group (Fig. 6b,c). Histological analysis confirmed the increase in TRAP<sup>+</sup> osteoclasts on the bone surface in response to SLA- and SB-originating Ti particles (Fig. 6d). It also further emphasized the dramatic bone resorption, especially in the SB groups, accompanied with the presence of inflammatory cells, fibrous tissue and new blood vessels (Fig. 6e). **Discussion** In the present study, we examined the release of Ti particles following ultrasonic scaling and their effect on both inflammatory responses in macrophages and osteoclastic bone resorption. The magnitude of the inflammatory response was assessed in relation to specific variables, including the type of implant surface from which the particles originated in addition to particle size and number.

Osteoclastogenesis and osteolysis were assessed both in vitro and in vivo. The primary outcome of the study is that introducing the Ti particles that are produced by an ultrasonic metal tip to mouse BMDMs elicits an inflammatory response. The presence of Ti particles in BMDM cultures induced a significantly higher inflammatory response than was observed following exposure to LPS. Remarkably, adding LPS to cultures that already contained Ti particles did not significantly increase the inflammatory response. These data further emphasize the dominant pro-inflammatory influence of the Ti particles that are released by US scaling. The observed effect of Ti particles on osteoclastogenesis and bone loss was in line with the observed inflammatory response. The secondary outcome of the study is that the physical properties of the particles differ according to the surface type from which they

originate. The particles produced by US scaling differ in their nanotopographies, chemical profiles and numbers but not in their average sizes. Importantly, our data suggest that both the

original implant type (which produces particles with different nanotopographies and chemical profiles) and the

number of particles significantly contribute to the induction of an inflammatory response.

The deleterious side effects of the by-products that are released by US of Ti implants has not been previously studied. The results of our study are in accordance with the literature on innate immune responses to Ti particles, which have been extensively investigated, mainly with regard for the particles shed by orthopedic Ti prostheses<sup>10,11,13,14,16</sup>. In our study, US scaling of Ti surfaces induced the release of particles that stimulated the secretion of pro-inflammatory cytokines. An increase in the expression of IL1 $\beta$ , IL6 and TNF $\alpha$ , among other cytokines, was accompanied by an increase in osteoclast formation and activity, which was either directly or indirectly induced via a paracrine effect on neighboring cells (e.g. osteoblasts). These cytokines have been repeatedly shown to trigger and/or amplify inflammation-induced bone loss<sup>13</sup>. In addition, it was recently suggested that chronic stimulation by Ti particles might lead to a state of oxidative stress and persistent inflammation<sup>17</sup>. A possible limitation of this study is that only short-term responses were assessed. We focused on acute responses to Ti particles and did not examine the long-term, chronic effects of exposure. However, the dramatic bone loss that was observed in vivo in the presence of Ti particles is unlikely to be reversible. Follow-up clinical studies are now needed to assess the long-term effects of US scaling of roughened Ti surfaces on particle-induced inflammation and osteolysis.

0 1500 3000 4500 6000

control L PS Ti Ti+LPS

Fold Increase

IL 1 $\beta$

0 2000 4000 6000 8000

control L PS Ti Ti + LPS

IL6

\*

\*\* \*\*

Control

\*

\*\*\*\*

Control Ti+LPS

0 5 10 15 20

control L PS Ti LPS+Ti

TNF $\alpha$

\*

\*\* \*\*

Control Ti + LPS LPS S Ti Control Ti+LPS LPS Ti Control Ti+LPS LPS Ti

Figure 2. Titanium particles released from SLA titanium implants induce inflammatory responses in macrophages. BMDMs were cultured for 24 hours with titanium particles that were released by ultrasonic scaling (Ti) and/or bacterial LPS (control). Saline was used as the control. IL1 $\beta$ , IL6 and TNF $\alpha$  expression levels were measured using RT-qPCR,

normalized to  $\beta$ -actin and expressed as a fold-change relative to the control level. The data are shown as the mean  $\pm$  SD of  $n = 5$  for each condition. These data are from a representative experiment out of 5 \* $p < 0.05$  versus control; \*\* $p < 0.05$  versus LPS and the control.

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Another limitation is the number of particles that were introduced into the tissue culture. The particle number was produced by 60 seconds of scaling, which we considered to be a reasonable duration for cleaning a partially exposed implant. In clinical practice, a portion of the released particles would find their way outside the tissue. The expected number of released particles trapped in the surrounding tissues would therefore be less than the number we tested. However, Giovanni et al. studied inflammatory responses in macrophages that were induced by an ultra-low concentration of nanoparticles and found that pro-inflammatory cytokines were significantly stimulated<sup>18</sup>. Similarly, our own experiments, in which we used only 10% of the particles that were released from one disc, also resulted in a significant increase in IL1 $\beta$  and TNF $\alpha$  expression in macrophages, and a significant bone resorption was observed in vivo when the same number of particles was added to membranes in our calvaria model. The present study also addresses the issue of mechanical cleaning of infected oral implants. Currently, only the effectiveness of bacterial biofilm removal and the damage to the implant surfaces has been described<sup>19,20</sup>. These changes depend on the initial topography of the implant and the type and mode of operation of the cleaning instruments<sup>19,20</sup>. The results of the study confirm the hypothesis that ultrasonic-produced particles elicit a strong immune and osteolytic response. To the best of our knowledge, this report is the first study to describe the potential biological consequences of performing US scaling on Ti implants. Orthopedic implants also release particles, due to mechanical wear. Future research should elucidate whether surface roughening also affects the number and physical properties of these wear particles and assess their impact on inflammation and aseptic loosening of the prosthesis. Materials and Methods Titanium Disc Characterization. Ti discs were made from grade 23 Ti6Al4V, prepared as cylinders that were 6 mm in diameter (28.27 mm<sup>2</sup> area) and 1.2 mm in height. The surface topography of the discs was machined

Figure 3. Titanium particles stimulate osteoclastogenesis in vitro. (a) TRAP staining (images and TRAP+area) of osteoclasts after 4 days of differentiation in the presence of LPS (0.01  $\mu$ g/ml), titanium particles or diluent only (control). Bar = 30  $\mu$ m. (b) The average number per well (N.Oc) and (c) the osteoclast area (mm<sup>2</sup>) in TRAP+cells after 4 days of differentiation. Data are shown as the mean  $\pm$  SD. \* $p < 0.05$  versus control; \*\* $p < 0.05$  versus LPS and control.

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(M), sand-blasted (SB) or sand-blasted and acid-etched (SLA) (AlphaBio Tec., Petah-Tikva, Israel). The surface of the discs is equivalent to the exposed area in a 3.75-mm diameter implant following  $\sim 2$  mm of vertical bone loss (Fig. 1). The Ti discs were analyzed to determine their nanoroughness using atomic force microscopy (AFM, NanoWizard III, JPK, Berlin, Germany) and their chemical composition using X-Ray photoelectron spectroscopy (Scanning 5600 AES/XPS multi-technique system, PHI, USA) prior to scaling.

Particle Generation and Characterization. Ti particles were generated using ultrasonic (US) scaling (Newtron Led, Satelec, Acteon, Marignac, France), adjusted to a frequency of 32 kHz. The resulting discs had one of the 3 previously described surface topographies. All particles were generated in a sterile environment. Each disc was submitted to US scaling for 60 seconds in distilled water (ddH<sub>2</sub>O) and then cleaned twice with ethanol and suspended in distilled water. The number of particles and their size distributions were evaluated using a

Figure 4. Inflammatory response dependent on the number and origin of titanium particles. BMDMs were cultured for 24 hours with: (a) Increasing numbers of titanium particles that were released by ultrasonic scaling of the SLA surface type. (b) 10% of the titanium particles that were released by ultrasonic scaling (Ti) of one disc from each implant surface: Machined (M), Sand-Blasted (SB), and SB/Acid-etched (SLA). (c) Same number of titanium particles (1293 particles/mm<sup>2</sup>) that were released by ultrasonic scaling (Ti) of each implant surface type: M, SB, SLA. IL1 $\beta$ , IL6 and TNF $\alpha$  expression levels were measured using RT-qPCR, normalized to  $\beta$ -actin and expressed as a fold-change relative to the control (no particles). The data are shown as the mean  $\pm$  SD of n = 5 for each condition. These data are from a representative experiment out of 5. The regression analysis formula and goodness of fit (R<sup>2</sup>) are shown (a). \*p < 0.05 versus M; \*\*p < 0.05 versus SLA and M. [www.nature.com/scientificreports/](http://www.nature.com/scientificreports/)

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Micro-Counter 1200 (Celeromics, Grenoble, France). Macro morphology was assessed using scanning electron microscopy (SEM, JSM-6300, JEOL Ltd, MA, USA) after the released particles were collected on carbon tape. An analysis of nanoroughness was performed using AFM. Because there is no established protocol for evaluating the nanoroughness of spherical particles, we used parameters similar to those utilized to analyze dental implant surfaces. Nine randomized line profiles were registered from particles originating from the 3 different types of surfaces. The average steepness was calculated for the x and y coordinates of the line profile every 10 nm along a linear distance of 500 nm. Linear distance differentiation (similar to Ra/surface area differentiation) was calculated using the Pythagorean formula to assess the increase between the actual line profile and the straight linear distance over 500 nm (one-dimensional measure). Peak-to-valley mean height (similar to Rz) was calculated as the average height difference between the highest peak and the deepest valley along the recorded 500 nm. The chemical composition of the particles was analyzed using energy-dispersive X-ray spectroscopy (EDS, JSM-6300, JEOL Ltd, MA, USA). Cell Culture. All procedures involving animals were carried out in accordance with the guidelines of the Tel Aviv University and were approved by the Institutional Animal Care and Use Committee (permit number M-015-047). Primary bone marrow-derived macrophages (BMDMs) were isolated from the femora and tibiae of adult C57BL/6J-RCC mice, as previously described<sup>21</sup>. Briefly, cells were cultured overnight in 6-well dishes at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> in alpha modified Eagle's medium ( $\alpha$  MEM, Life Science Technology, NY, USA). After 24 hours, the non-adherent fraction was cultured in 10-cm non-culture-treated dishes containing  $\alpha$  MEM supplemented with 10% fetal bovine serum (FBS, Rhenium Ltd, Modi'in, Israel) and 100 ng/ml macrophage colony stimulating factor (M-CSF). M-CSF was obtained from CMG (14–12) cells as previously described<sup>22</sup>. The resulting adherent BMDMs were collected and seeded in 6-well plates (106 cells/well). Ti particles with different origins, bacterial

lipopolysaccharide (LPS, 0.01  $\mu$ g/ml, used as the positive control), or diluent only (control) were added to the cultures, and the cells were incubated for 24 to 48 hours as indicated. This dose of LPS has been reported to induce a significant inflammatory response in primary macrophages<sup>15</sup>. For the osteoclastogenesis assay, BMDM, which are the same as preosteoclasts in vitro, were plated in 96-well plates (7,000 cells per well) in standard medium supplemented with 20 ng/ml M-CSF and 50 ng/ml RANKL (R&D Systems, Minneapolis, MN, USA), replaced every 2 days, as previously described<sup>21</sup>. Treatments (either LPS or titanium particles) were added after two days of incubation. On the 4th day, cells were stained using a TRAP kit (Sigma-Aldrich, St. Louis, MO, USA), and multinucleated (< 3 nuclei) TRAP-positive cells were defined as osteoclasts. Images were acquired at an original magnification of  $\times 4$  (Evos FLC, Life Technologies, MS, USA). Osteoclast number and total osteoclast area were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Apoptosis. For analysis of apoptosis, macrophages were harvested by trypsinization 24 hours after medium change, and stained with FITC-conjugated Annexin V and propidium iodide for 15 min, according to the manufacturer's instructions (MBL, Nagoya, Japan). Cells positive for Annexin V and propidium iodide were recorded using flow cytometry (Gallios, Beckman Coulter, Indianapolis, IN, USA). The results were analyzed using Kaluza software (Beckman Coulter, Indianapolis, IN, USA). Figure 5. Schematic of exposed implant surfaces. Micro-CT 3D rendering of a 3.75 mm diameter implant with a putative horizontal bone loss of 2.05 mm (arrows). The resulting exposed region (light gray) corresponds to a total implant surface of 28.27 mm<sup>2</sup>.

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Protein analysis, RNA Isolation and RT-qPCR. After incubation, the supernatant was collected and secreted protein amounts of IL1 $\beta$ , IL6 and TNF $\alpha$  were measured using multiplex assay and expressed in MFI units (Multiplex Fluorescent Immunoassay, ProcartaPlex Multiplex Immunoassay, eBioscience, San Diego, CA, USA). After supernatant collections, macrophages were washed with sterile PBS, and RNA was extracted using Tri-RNA Reagent (Favorgen Biotech Corp, Kaohsiung, Taiwan). The 260/280 absorbance ratio was measured to verify RNA purity and concentration. cDNA was produced using a High Capacity cDNA Reverse Transcription Kit (Invitrogen, Grand Island, NY, USA), and real-time PCR was performed using Kapa SYBR Fast qPCR (Kapa Biosystems, Wilmington, MA, USA) on a StepOne real time PCR machine (Applied Biosystems, Grand Island, NY, USA). We examined the expression of IL1 $\beta$ , IL6 and TNF $\alpha$ , which are established markers of macrophage inflammation. The primer sets were as follows: F-GAAATGCCACCTTTTGACAGTG and R-TGGATGCTCTCATCAGGACAG for mouse IL1 $\beta$ ; F-TAGTCCTTCCTACCCCAATTTCC and R-TTGGTCCTTAGCCACTCCTTC for mouse IL6; and F-TCTTCTCATTCCTGCTTGTGG and R-GGTCTGGGCCATAGAACTGA for mouse TNF $\alpha$ . The reaction was subjected to 40 cycles of amplification using the following program: 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 25 s. The relative mRNA expression levels

Figure 6. Titanium particles originating from US scaling of dental implants induce osteolysis in vivo. Titanium particles that originated from US scaling of SB and SLA discs were inserted into fibrin membranes and implanted onto the calvaria of 6 mice per group. Animals were then sacrificed after 5 weeks. (a) Representative  $\mu$ CT images of the calvaria are shown. The region of interest (ROI) is represented as dark gray, and the resorption pits are represented as red. (b) Pit Resorption Volume (PRV,  $\mu$ m<sup>3</sup>) and (c) PRV are shown

relative to bone tissue volume inside the ROI (PRV/TV, %). The data are expressed as the mean  $\pm$  SD, n = 6. \*p < 0.05 versus control (membrane with no particles); \*\*p < 0.05 versus SLA and control. (d) Histological TRAP-stained sections demonstrating the increase in lining osteoclasts (blue arrows). (e) HE staining demonstrating the presence of blood vessels (\*), inflammatory cells and fibrous tissue, especially in the SB group. Original magnification  $\times 40$ .

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of the selected genes were normalized to the level of  $\beta$ -actin, which was amplified using the following primers: F-GTCACCCACACTGTGCCCATC and R-CCGTCAGGCAGCTCATAGCTC. Animal Model and Micro-Computed Tomography ( $\mu$ CT). Membrane preparation. Titanium particles (generated as described before) were embedded in fibrin membranes, which were used as scaffolds to localize the titanium particles. Fibrin membranes were prepared in 48-well plates by mixing fibrinogen from bovine plasma with thrombin from bovine plasma (Sigma-Aldrich, St. Louis, MO, USA). Membranes with no particles were prepared as controls.

Surgical insertion model. After anesthesia, the skin of C57Bl/6J-Rcc female mice was shaved and disinfected at 10 weeks of age. The parietal bones of the mice were exposed via a 10-mm incision in the nape area, and the periosteum was removed using a periosteal elevator. Membranes with titanium particles with different surfaces were inserted to cover both parietal bones. The surgical incision was then closed using nylon monofilament surgical sutures (5/0). In the sham controls, membranes with no particles were inserted and the incisions were closed. All groups consisted of 6 animals. After a follow-up period of 5 weeks, the animals were sacrificed and the skull of each mouse was removed, fixed for 24 hrs in 4% phosphate-buffered formalin followed by ethanol 70%. All specimens were scanned and analyzed using a  $\mu$  CT system ( $\mu$  CT 50, Scanco Medical AG, Switzerland). Scans were performed at a 10- $\mu$  m resolution in all three spatial dimensions, with 90 kV energy, 88  $\mu$  A intensity and 1000 projections at a 1000 msec integration time. The region of interest (ROI) was defined as two 3.7-mm circles in the center of the parietal bones. The mineralized tissues were differentially segmented using a global thresholding procedure<sup>23</sup>. A custom-made algorithm based on Image-Processing Language (IPL, Scanco Medical) was developed to isolate the resorption pits, defined as unmineralized pits that were 10- to 40- $\mu$  m deep on the bone surface. The measured resorption volume was limited to a 40  $\mu$  m depth because beyond that, the resorption pit was connected to the internal diploe. Morphometric parameters were determined using a direct 3D approach<sup>24</sup> and included the total volume of the bone resorption (Pit Resorption Volume, PRV,  $\mu$  m<sup>3</sup>) and bone tissue volume inside the ROI (TV,  $\mu$  m<sup>3</sup>), which was used to determine the PRV/TV (%).

Histological analysis. Following  $\mu$  CT scanning, the specimens underwent decalcification in 10% EDTA, dehydration in graded alcohols and embedding in paraffin. Each calvaria was serially sectioned to a thickness of 5  $\mu$  m in the coronal plane. Each section was sampled three times, 0.5 mm apart. Half the sections were stained with hematoxylin-eosin (HE) and serial sections with tartrate-resistant acid phosphate (TRAP, Sigma). Images were acquired using an Olympus BH2 microscope (Olympus, Japan), attached to an Olympus DP70 camera. All images were acquired using a  $\times 40$  lens and further enlarged 10-fold by the camera (final magnification  $\times 400$ ).

Statistical analysis. Values are expressed as the mean  $\pm$  SD unless otherwise indicated. Statistical analyses were performed using GraphPad Prism 7.0 (La Jolla, CA, USA). As all presented data typically display a normal distribution, analysis of variance (ANOVA) and Tukey's post hoc test for multiple group comparison were used. Differences between groups were defined as significant at  $p < 0.05$ .

Summary. Ti particles were introduced to BMDMs by applying an ultrasonic metal tip to titanium discs. This elicited an inflammatory response and resulted in dramatic bone loss. The Ti particles induced a significantly higher inflammatory and osteoclastogenic response than was induced by LPS. The physical properties of the particles that were derived from different implant surface types differed in their nanotopographies, chemical compositions and numbers but not their average sizes. The implant surface type and the number of particles significantly contributed to the induction of inflammation. This study suggests that the biological consequences of implant cleaning procedures have a significant clinical impact. Thus, evaluations of treatment results should concentrate not only on the efficiency of the cleaning and the damage caused to the implant surface but also on the quantity and properties of the released particles.

### 32. What is incorrect about extractions?

- A periosteal elevator is an instrument that can be used to perform an atraumatic tooth extraction.
- The periodontal ligament does not need to be removed because the socket will heal by itself with no complications.
- A bone graft can be placed to assist the socket in healing and improve the bone density for an implant to be placed a few months later.
- A “cavitation” is a lesion in the bone that may be a result of toxic elements left in the bone socket after a tooth extraction.

#### Periosteal Elevator as an Aid to Atraumatic Extraction: A Comparative Double Blind Randomized Controlled Trial

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**Abstract** Aim The aim of this study was to evaluate the efficacy of periosteal elevators in single rooted nonsurgical tooth extractions. Materials and methods A double blind, randomized controlled clinical trial of 100 patients requiring nonsurgical single rooted tooth extractions was performed. The subjects were randomized into the experimental group (underwent extractions with periosteal elevator and conventional extraction forceps) or into the control group (subjects underwent extractions using periosteal elevator and conventional extraction forceps). Pain was assessed using visual analogue scale all throughout 7 days postoperatively. Gingival laceration, duration of surgery, number and frequency of analgesics consumed and complications (if present) were also noted. Results On inter-group comparison, all the parameters were statistically significant in control group ( $p < 0.05$ ).

Also on pre and post-operative inter-group comparison, statistically significant pain reduction was noted in experimental group (52.8 %) whereas pain increased in control group (65 %). Conclusion The results of this study suggest that use of periosteal elevator may be helpful in reducing post extraction discomfort.

**Keywords** Atraumatic Extraction Periosteal Elevator Complications Gingival Laceration Introduction

The specialty of maxillofacial surgery has made tremendous strides in the past few decades encompassing such diverse fields as craniofacial surgery, microvascular reconstruction, etc. But the most commonly performed procedure by maxillofacial surgeons in many countries continues to be exodontia, comprising non-surgical routine tooth extractions as well as impacted tooth removal. Improvements in local anaesthetics as well as its delivery armamentarium have resulted in painless extractions but the fear of post extraction pain deters many patients from undergoing this procedure [1–6]. Soft tissue trauma is one of the reasons for post extraction pain and various techniques have been tried to reduce this component's contribution. With the advent of implantology, atraumatic extraction has come into vogue again and proponents of periosteal elevator have claimed that it not only reduces soft tissue injury but also aids in salvaging the bony integrity of the socket as well. There are not many studies or case reports regarding the usage of periosteal elevator in exodontia, so we decided to conduct a prospective, double blind, randomized controlled trial to

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J. Maxillofac. Oral Surg. (July–Sept 2015) 14(3):611–615 DOI 10.1007/s12663-014-0723-8 compare the efficacy of periostome in nonsurgical single rooted tooth extractions.

Materials and Methods

A randomized, double blind, controlled clinical trial was performed in 100 patients. All patients underwent the nonsurgical extractions of single rooted teeth between October, 2012 and March, 2013. The study was approved by the Research and Ethics Committee of the institution. Before enrolment, the objectives, implications and possible complications of this clinical trial were explained to all the patients and informed consent was obtained. Inclusion criteria included patients above 14 years of age requiring nonsurgical removal of either maxillary or mandibular single rooted tooth. Exclusion criteria consisted of refusal of informed consent, patients who were taking drugs which interfere with pain response, allergy to ibuprofen, pregnancy and history of intake of analgesics up to 10 days prior to extraction. Random allocation of groups was done using computergenerated randomization process to two groups. The patients did not receive any financial compensation for participating in the study. Tooth extractions were carried out aseptically under local anaesthesia (2 % lignocaine with 1:2,00,000 adrenaline) and post extraction instructions were given to each patient. In the control group, after clinical assessment of tooth to be extracted periosteal elevator was used for reflecting the gingiva to expose the cemento-enamel junction and the extraction was carried out using conventional forceps (Fig. 1). In the test group, after clinical assessment of tooth to be extracted Amron periostome with blade attachments was held in modified pen grasp and inserted at 20 degrees to the long axis of tooth into the gingival sulcus (Figs. 2, 3). It was used to sever the cervical gingival attachment fibres first and then proceed several millimetres into periodontal ligament space and inclined first mesially and then distally tangential to root surface. Once the access was obtained,

the instrument was gradually advanced into the PDL space repeating the same motion until two-thirds of the distance towards the apex of root was reached. Then tooth was extracted using extraction forceps exerting rotational force in a coronal direction [7–10] (Fig. 4).

Fig. 1 Postextraction gingival laceration by conventional method

Fig. 2 Operating angulation

Fig. 3 Angulation of periostome to the tooth to be extracted

Fig. 4 Post extraction socket using periostome

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Ibuprofen 400 mg was given immediately after completion of extraction and 1 Tab SOS later as analgesic [11, 12]. No other postoperative medication was prescribed. Patients were also instructed not to seek any medical help elsewhere for postoperative problems, if any, but to report to our department. They were followed-up for a minimum period of 1 week for evaluation of wound. During the preoperative phase, pain was assessed using visual analogue scale before the administration of LA [13, 14]. During the intraoperative phase, duration of procedure was calculated from the onset of local anaesthesia till the completion of tooth extraction. Immediate post-op, complications, if any, were recorded. Gingival lacerations were graded using the following scale:

Grade 1 Grade 2 Grade 3 Grade 4

Length 0–5 mm 5–10 mm [1 cm Torn Gingiva Depth Abrasion Partial Complete depth  
Postoperatively, patients were instructed to measure the intensity of postoperative pain throughout the period of 7 days (3rd h, 6th h, 24th h, 7th day) on visual analogue scale [13, 14]. They also had to record the number and frequency of analgesics consumed and any other complications. The following variables were also gathered: age, sex, tooth, mobility grade and operator. All the patients and investigator were blinded to avoid bias. Data on 100 subjects (49 males and 51 females) was entered in Microsoft Excel and subjected to statistical analysis using SPSS version-11 statistical package. The critical level of significance was set at  $p < 0.05$ . Demographic data was analysed using Chi square tests. Student's t tests were used for independent samples and Karl Pearson correlation was used to calculate the scientific data and the association between the various variables. To compare the two groups regarding different parameters, repeated measures ANOVA tests were used.

#### Results

One hundred patients were enrolled, 4 (2 each in control and test group) were lost because they did not attend follow-up visits. The results were based on the analysis of 96 participants with 48 patients in each group. Table 1 and Graph 1 shows the duration of procedure in control group which is significantly greater than test group ( $p < 0.001$ ). Analgesic consumption was also more in the control group. Pain reduction in test group was significantly greater than control group on inter-group comparison ( $p < 0.05$ ). Gingival lacerations were also more in the control group— $p < 0.05$ . Table 2 and Graph 2 shows the intra-group variance in test and control groups in reference to pain. There is a significant reduction in pain in test group ( $p < 0.05$ ) whereas in control group there is a significant increase in postoperative pain as compared to preoperative pain ( $p < 0.05$ ). It was also found that there was significant difference of complication rate in test and control groups ( $p < 0.05$ ). Complications like mild pain on 7th day etc. were

Table 1 Distribution of mean and SD between test and control group (t test for two independent groups)

Group N Mean  $\pm$  SD Std. error mean

p value

Duration of operation Control group 50  $12.8116 \pm 7.393823$  1.045644 0.000 Test group 50  $5.78010 \pm 4.043751$  0.571873  
Frequency of analgesic consumed Control group 48  $12.50 \pm 5.120$  0.739 0.000 Test group 48  $4.44 \pm 3.135$  0.452  
No. of analgesics consumed Control group 48  $2.21 \pm 0.617$  0.089 0.000 Test group 47  $1.40 \pm 0.577$  0.084  
Pain reduction Control group 48  $-0.7083 \pm 2.20694$  0.31854 0.002 Test group 48  $0.8854 \pm 2.65426$  0.38311

Gingival laceration grade Control group 50  $1.2000 \pm 0.90351$  0.12778 0.000 Test group 50  $0.2000 \pm 0.63888$  0.09035

Table 2 Pain reduction in control and test group

Mean  $\pm$  SD Std. error mean

p value

Test group Preoperative pain

$1.63 \pm 2.506$  0.358 0.025

Postoperative pain

$0.765 \pm 0.6778$  0.0968

Control group Preoperative pain

$1.08 \pm 2.019$  0.291 0.031

Postoperative pain

$1.792 \pm 1.0408$  0.1502

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significantly more in control group than test group ( $p < 0.05$ ) (Graph 3). No association of different parameters with the grade of mobility of tooth was observed.

#### Discussion

Traditional extraction methods have a history of not only producing postoperative pain but also damaging the hard and soft tissues surrounding the tooth [15]. Conventional extraction techniques either elevate the tooth by leveraging against the interproximal bone resulting in damage to the interproximal bone or use of forceps to luxate the tooth from its socket which often results in reshaping of the socket or alveolus [10]. This leads to difficulty in maintaining the socket integrity due to hard tissue damage and thus making future prosthetic replacement difficult. Also Bortoluzzi et al., Sjögren et al., and Al Khateeb, have conducted studies on postoperative pain in exodontia and observed it as the most common complication [1–3]. Many other complications are also prevalent in exodontia cases due to the conventional methods. Adeyemo et al. [16] have mentioned about presence of alveolitis in 11 % sockets and mild pain in 12 % cases. Bortoluzzi et al. [17] in their study observed an incidence of 0.6 % (2 cases each) for both alveolar infection and dry socket. Schropp et al. [18] in their study on bone healing of extracted socket mentioned about the major chances of bone loss at extraction site 1 year after tooth extraction. In another study by Adeyemo et al. [19], they discussed about the various pre-operative complications such as accidental crown, root or alveolar bone fractures which often lead to healing complications and even increased time of extraction due to such complications leading to disturbance in healing. Venkateshwar et al. found tooth fracture, trismus, fracture of cortical plates and dry socket to be the most common complications while wound dehiscence, and postoperative pain were the rare complications and luxation of adjacent teeth, fracture of maxillary tuberosity and displacement to adjacent spaces among the rarest complications encountered during tooth extraction [20]. Similar outcomes to the above studies were observed in our study in the control group where postoperative pain, buccal cortical plate fracture, bleeding till 2nd day, dry socket, apical third root fracture and erythematous margins were observed. Even the oral health-related quality of life following nonsurgical routine tooth extraction deteriorates with conventional method of extraction as in control group of our study [21].

Marco Cicciu` et al. [22] in their study have mentioned that extraction of teeth was not affected by the amount of strength applied or the quality of bone surrounding the tooth but is more technique sensitive. At the same time it is believed that an excessive force which exceeds expansion of socket results in fracture of alveolus specially in elderly patients in whom bone is dense and sclerotic. To avoid the above mentioned problems and following a more technique sensitive approach, we need to proceed with “atraumatic extraction technique”. Atraumatic extraction preserves bone, gingival architecture and allows for option of future or immediate implant placement. There are a variety of tools available for a minimally invasive technique of tooth extraction such as Easy X-Trac system [4, 23], physics forceps [9, 24] and periostomes. In our study, we have used periostome as a means of atraumatic extraction. This instrument helped in removing firm tooth and retained roots without damaging the surrounding thin alveolar plates of bone and minimally lacerating the soft tissue as well. This may aid in providing a completely supportive environment for both immediate and delayed implant placement. Thus, the above concept supports the biomechanical rationale for atraumatic extraction. Also periostome seemed to be helpful in maintaining the soft and hard tissue architecture specially in extracting endodontically treated teeth and crown fracture cases. It aids in removing the tooth without damaging the osseous housing [10]. Similar findings were noticed in our study with the maximum number of buccal cortical plate fractures and apical third root fractures occurring in control group as compared to the test group. Periostome provided the opportunity to remove such teeth without reflection of flap and thus avoiding the need of mucoperiosteal flap and exposure of bone. This may be helpful in leaving the shape of extracted socket undisturbed and alveolus intact. In the test group where periostome was used, duration of surgery, frequency and number of analgesics consumed, pain reduction and gingival laceration favoured the use of this instrument for extraction. Even with some of the drawbacks of our study (since it is a new study we could not compare it with any other study and also because the extractions were not performed by a single operator), we are of the opinion that use of periostome in single-rooted tooth extractions gives a superior result compared to extractions carried out using the traditional periosteal elevator.

Bone manipulation procedures in dental implants Yuvika Mittal, Govind Jindal, and Sandeep Garg

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Abstract The use of dental implants for the rehabilitation of missing teeth has broadened the treatment options for patients and clinicians equally. As a result of advances in research in implant design, materials, and techniques, the use of dental implants has

increased dramatically in the past two decades and is expected to expand further in the future. Success of dental implants depends largely on the quality and quantity of the available bone in the recipient site. This however may be compromised or unavailable due to tumor, trauma, periodontal disease, etc., which in turn necessitates the need for additional bone manipulation. This review outlines the various bone manipulation techniques that are used to achieve a predictable long-term success of dental implants.

Keywords: Bone expansion, bone grafts, guided bone regeneration, maxillary sinus lift

**INTRODUCTION** The loss of tooth can be psychologically traumatizing. Attempts to replace teeth have been seen even in ancient civilizations. What makes implant dentistry unique is the ability to achieve this goal, regardless of the atrophy, disease, or injury of the stomatognathic system. To satisfy the ideal goals of the implant dentistry, the hard and soft tissues need to present ideal volumes and quality. If inadequate bone exists, several surgical techniques may be used to reconstruct the deficient ridge for implant placement.[1] The bone manipulation techniques are capable of manipulating the one's bones to alter their density to make them extremely durable and strong. These techniques mobilize vital bone with plastic bending, shaping, or condensation of tissue as a bone flap or bone-periosteal flap.[2] These result in contour or dimensional changes, while preserving bone integrity and viability. The concept is to manipulate the residual bone to create an intrabony cavity with a wider base or taller roof that heals like an extraction site, with access of mesenchymal stem cells and the normal wound healing mechanisms. The morphology of bony defect is an important consideration in the selection of a method for ridge manipulation. The fewer the number of remaining bony walls, the greater is the need for osteopromotive techniques.[1] Current bone manipulation techniques include inlay and onlay grafting, guided bone regeneration (GBR), bone expansion, bone splitting osteotomy, and different fixation devices such as bone screws, pins, titanium

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mesh, different augmentation materials, and different barrier membranes.[3]

**PATTERN OF BONE LOSS** The alveolar bone loss is known to occur at a rapid rate during the 1 year after tooth extraction and may continue for years. Within the 1 year of the tooth loss, there is a 25% decrease in the width of the crestal bone and a 40% decrease in the bone width occurs within the first 1–3 year after tooth extraction, resulting in a labial plate of bone that is located lingual of its original location. Preservation or recontouring of the labial appearance of the alveolar process is one of the keys to optimal implant esthetics and long-term results.[4]

**AVAILABLE BONE** A multidimensional assessment of the available bone is the most important factor necessary for a sound treatment planning ensuring longevity and function of implant supported prosthesis. Determinants of available bone are:

Height Width Length Angle Crown/implant ratio.

The minimum implant length (i.e., bone height) in an ideal bone density situation for predictable success is 10 mm. Allowing a margin of 2 mm from the vital landmarks such as inferior alveolar canal is recommended. Available width is defined as the distance between the buccal and the lingual plates, measured at the crest. Each 1 mm increase in diameter

increases the surface area by about 20–30%, therefore, increasing diameter effectively; decreases crestal stress. Thus, implant diameter is much more critical than its length.[1] RATIONALE FOR BONE MANIPULATION Dental implants are the treatment of choice for the replacement of missing teeth nowadays. However, the placement of implants in the alveolar bone remains a challenge for most of the clinicians because of the resorption of the residual ridge resulting in the insufficient bone volume in one or more dimensions. Various surgical techniques to augment the thin ridges not only increases the morbidity but also results in the increase of expenditure involved as well as the time taken. All these factors act as determinants for the acceptance of the treatment plan by the patient. Need of the hour is to review the various bone manipulation techniques developed over the years and use a suitable conservative technique. Bone is a biologically privileged tissue in that it has the capacity to undergo regeneration as a part of repair process.[5] Adequate bone volume prerequisites the implant therapy and proper esthetic result. Inadequate alveolar bone height and width often require bone manipulation procedures performed before, at the time of, or after the implant surgery. There are various techniques that had been described for the bone manipulation [Flow Chart 1].

#### SURGICAL PROCEDURES

**Bone expansion technique** It is defined as the manipulation of the bone to form a receptor site for an implant without the removal of any bone from the patient.[6] The objective is to maintain the existing soft bone by pushing the buccal bony

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plates of the residual ridge laterally with minimal trauma. This technique takes the advantage of the softer bone quality found in Types III and IV maxillary bone by relocating the alveolar bone rather than losing the precious bone by drilling [Figure 1].[7] The most common anatomic area in which ridge expansion is performed is in the narrow anterior maxilla, followed by posterior maxilla and then the anterior and posterior mandible, respectively. Width of the residual alveolar crest should not be <4 mm to be able to insert the round osteotomes:

For reducing maxillary undercuts For changing the emergence angulation For expanding the buccal or labial bone esthetic reasons.

Bone expansion can be done by means of osteotomes.[8] Osteotomes are a special set of instruments developed to form or shape bone in preparation for the placement of dental implants. It increases the width for implant placement and allows immediate placement of implants in narrow ridges at the time of expansion. This technique also allows for greater tactile sensitivity. In spite of so many advantages, osteotomes have certain disadvantages too. It is based on palm-held design that can be problematic to use in the posterior maxilla due to limited mouth opening. If the cortical plates of the bone are fused due to atrophy, the osteotome technique may not be effective.

**Alveolar ridge splitting technique** This technique can be used to augment the atrophic maxilla and mandible before the implant placement. This method was introduced by Dr. Hilt Tatum in the 1970s and was commonly referred to as ridge splitting or bone spreading technique.[9] Gaining access to a ridge that is <3 mm wide requires splitting the buccal and

palatal bone flaps with a scalpel first by separating two cortices through its cancellous bone. This technique is employed in cases where there is an insufficient width to utilize round osteotomes. This procedure provides a quicker method wherein an atrophic ridge can be predictably expanded and grafted with bone allografts, eliminating the need for a second donor site and a second stage surgery [Figure 2]. Ideal sites demonstrate a knife-edge ridge that widens further apically, and that consists of adequate cortical thickness but with some degree of interpositional lamellar bone. The anterior region of the maxilla usually meets these demands, whereas mandibular sites usually do not. When there is adequate height of residual ridge, 8–10 mm of bone between the crest of the ridge and opposing landmark but inadequate buccolingual width, ridge splitting is an option. This technique enables problem-free widening from 2 to 5 mm. Narrow edentulous alveolar ridges <4 mm wide can be expanded by the following means:

**Single stage ridge-split procedure:** In this procedure, entire edentulous bony segment is opened like an envelope to receive the implants. The pilot drills are used after the desired ridge widths have been reached and the implants inserted  
**Two stage ridge split technique:** This is indicative when enough primary stability is not achieved. With this approach, the location of greenstick fracture is predetermined, and blood supply to the lateralized buccal segment remains intact.

The osteoperiosteal flap ridge-split procedure is performed for horizontal augmentation of narrow ridges that otherwise would not be suitable for implant placement. This technique consists of splitting the vestibular and the buccal cortical plate and further opening the space with osteotomes.[10] This technique is advantageous as it does not require waiting period of 4–6 months for bone consolidation before the implant placement. In addition, it decreases the morbidity since it avoids a second surgical donor site for bone harvesting.

However, this procedure can only increase the buccolingual bony dimension and is

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Autogenous bone grafts

Allografts

Alloplasts

Xenografts

not applicable if there is sufficient bone height. Furthermore, implementing the technique on atrophic ridges <3 mm wide may result in unfavorable bone fractures that lead to bone resorption. Therefore, it is demonstrated that the ridge splitting technique is effective in longitudinal expansion of the alveolar ridges.

**Bone grafting procedures and guided bone regeneration** The gold standard of bone grafting materials is autografts. Autografts are obtained from the same patient, taken from one site and placed in another site and forms the bone by the process of osteogenesis and osteoinduction. Autograft materials are obtained intraorally from edentulous areas such as maxillary tuberosity, mandibular symphysis, and mandibular ramus. Extraoral autografts are obtained from iliac crest, rib, tibia, and calvarium. The advantages of autograft bone material are that it maintains bone structures such as minerals, collagen, and viable osteoblasts and bone morphogenic proteins (BMPs). The best success rates in bone grafting have been achieved with autografts because these are essentially living tissues with their cells intact [Figure 3]. It is osteoinductive/conductive, sterile,

biocompatible/nonimmunogenic, easy to manipulate, and readily available from adjacent or remote sites. The microscopic architecture is perfectly matched. The main disadvantage of autografting is that it has to be harvested from a secondary (either intra- or extra-oral) site, which usually means more complicated surgery and higher morbidity. An allograft is a tissue graft between individuals of the same specimen but of nonidentical genetic composition. The source is usually cadaver bone, which is available in large amounts. This bone has to undergo many different treatment sequences to render it neutral to immune reactions and avoid cross contamination of host diseases. In practice, fresh allogenic bone is rarely used because of immune response and the risk of disease transmission. Human bone material in the form of freeze-dried bone or demineralized freeze-dried bone has been used widely in implant dentistry. A wide range of grafts is available, which may be particulate, thin sheets of cortical plate, or much larger bone blocks. Allografts have been used as an alternative but have little or no osteogenicity, increased immunogenic, and resorb more rapidly than autogenous bone. Allograft bone is a useful material in patients who require bone grafting of a nonunion type but have inadequate autograft bone. It is predominantly used as a scaffold for bone repair and is resorbable. Alloplastic bone grafts are synthetic materials that have developed to replace human bone to avoid transmitted diseases such as HIV, bovine spongiform encephalitis, or hepatitis. They are biocompatible and osteoconductive materials. The most common types of alloplasts used are calcium phosphates, bioactive glasses, and biocompatible composite polymers. Moreover, the main disadvantage of alloplasts is that they are unpredictable in allowing bone formation; therefore, particles can be uncounted within the grafted site. Furthermore, the natural bioceramics are calcium carbonate materials, with similar to the natural bone hydroxyapatite structure. Advanced synthetic bioactive resorbable bone graft materials having similar chemical and mechanical properties as the host bone, can provide the means to modify existing bone topography. Hydroxyapatite is available in a variety of forms. The most commonly used nonresorbable form becomes embedded in newly formed fibrous tissue and bone, and the resulting tissue combination is a less than ideal implant bed. The use of alloplastic grafting materials on their own is not routinely recommended. Hydroxyapatite and other bone substitutes require further clinical research and should not be used on their own as grafting material until their efficacy is evidence-supported. Xenografts are graft materials derived from the inorganic portion of animal bones; the most common source is bovine, the removal of the organic component is processed to remove their antigenicity, whereas the remaining inorganic components provide a natural matrix as well as an excellent source of calcium. The disadvantage of xenografts is that they are only osteoconductive, and the resorption rate of bovine cortical bone is slow.

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Guided bone regeneration

Growth factors

Currently, Bio-Oss and Bio-Gide are widely used as dental xenograft materials. Bio-Oss is a xenograft consisting of deproteinized, sterilized bovine bone with 75–80% porosity and a crystal size of approximately 10  $\mu\text{m}$  in the form of cortical granules; it has a natural, nonantigenic porous matrix and is chemically and physically identical to the mineral phase of human bone; it has been reported to be highly osteoconductive and to show a very low

resorption rate. The organic material is completely removed to leave the mineralized bone architecture, which renders it nonimmunogenic and presumably safe from the possibility of infection. A more recent study demonstrated a favorable long-term tissue response to BioOss particles with mainly woven immature bone shown at 20 months, which was replaced with lamellar bone with time. Bio-Oss is becoming increasingly popular for the use in bone grafting in implant dentistry and is often used in combination with Bio-Gide . Bio-Gide is a membrane made of collagen which facilitates planned soft tissue management during augmentation. Bio-Gide is composed of highly purified natural collagen from pigs which has a natural bilayer design and has native collagen for soft tissue compatibility and forms a barrier for undisturbed bone regeneration. Studies have shown Bio-Gide to allow successful bone regeneration in combination with Bio-Oss and provide a barrier function lasting several months. It is also known as “membrane protected bone regeneration.” The concept of GBR implies the use of cell-occlusive membranes for space provision over a vertical or horizontal defect, promoting the in-growth of osteogenic cells while preventing migration of undesired cells from the overlying soft tissue. It also effectively stabilizes the blood coagulum and thereby allows for faster healing to occur. This technique can be used before or at the same time as implant placement. Barrier membranes may be nonresorbable (e.g., expanded polytetrafluoroethylene) or resorbable.[11] Although nonresorbable has shown the most bone volume gain, they are associated with higher incidence of complications such as membrane exposure due to soft tissue dehiscence [Figure 4]. Various growth factors have widely been tested in animal models. Of these, bone BMPs require special mention as they induce osteogenic precursor cells into osteogenic cells and have shown tremendous bone growth in many animals and also human clinical studies. Other growth factors besides BMPs that have been implicated during bone regeneration are also being investigated, including platelet-derived growth factor, transforming growth factor- $\beta$ , insulin-like-growth factor-1, vascular endothelial growth factor, and fibroblast growth factor, among others.[12]

Maxillary sinus lift procedures Currently, two main approaches to the maxillary sinus floor elevation procedure can be found in the literature. The first approach, lateral antrostomy, is the classic and the more commonly performed technique originally described by Tatum. This technique is often used as a preimplant procedure when residual alveolar ridge is inadequate to a point where initial implant stability is compromised. More recently, Summers advocated a second approach, the crestal approach, using osteotomes.[13] The crestal approach is considered to be a more conservative method for sinus floor elevation. In this technique, maxillary floor is fractured, the sinus membrane is elevated through an implant site with the use of osteotomes [Figure 5]. The crestal technique has the advantage that it improves the density of the maxillary bone. It also has the potential for the use of less autogenous grafting material. The disadvantage of this approach is that the initial implant stability is unproven if the residual bone height is <6 mm. The chances of achieving a sufficiently high elevation with osteotome technique are limited. Other alternative techniques used nowadays in sinus lift procedures include the hydraulic pressure technique, endoscopically controlled technique called as subantrosopic laterobasal sinus floor augmentation, antral membrane balloon elevation technique, dentium advanced sinus kit technique, grind-out technique, and wall-off technique.[14]

Alveolar distraction osteogenesis

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Use of zygomatic implants

Use of tilted implants

Use of basal implants

Distraction osteogenesis of the edentulous alveolar ridges may be considered an alternative to many other surgical techniques such as alloplastic graft augmentation, autogenous onlay bone grafting, and GBR. Alveolar distraction is now widely used for treating severe forms of alveolar ridge atrophy.[15] In this technique, a defect is created when two bone segments are slowly separated under tension. One week after osteotomy and distractor placement (latency period), distraction of segments is advanced at a rate of 0.5–1 mm/day until the desired separation is reached. A consolidation period of 5 days/mm of the space created should be maintained before device removal and implant placement. It allows for a vertical bone gain of 3–20 mm without the use of graft material, and additional mucosal grafting is not required as the soft tissue follows bone distraction [Figure 6]. Currently, vertical distraction osteogenesis of alveolar bone can be performed with three distraction systems:

Central application device (e.g., LEAD system) Eccentric application of the device (e.g., TRACK distractor, Martin GmbH, and Co., Germany) Distraction by an implant (e.g., DIS-SYS distraction implant; Sis Inc., Klagenfurt, Austria).

One of the main problems in alveolar distraction is the accurate control of the direction. When the transport segment is relatively long (more than about 2 cm), it may be difficult to achieve controlled osteogenesis using only one distraction device. There is the possibility of tilting in the longitudinal axis of the distraction. In such cases, one solution is to use two distractors for each transport segment. Used singly, intraosseous distractors effectively control the movement of the transport segment in only one-dimension, which is the axis of distraction. The use of two distractors, one at each end of the segment, resolves this problem by ensuring that the segment stays parallel to the alveolar ridge.[16]

Other alternatives

Zygomatic implants are a suitable alternative for the treatment of severe posterior maxillary atrophy. Three different surgical techniques exist for placing zygomatic implants: The sinus window technique (classic), the sinus slot technique, and the procedure for extrasinus zygomatic implants. The classic sinus window technique consists of exposing the frontolateral face of the zygomatic bone and creating a 10–35-mm window in the sinus to visualize the implant trajectory. The sinus slot technique improves visualization of the implant positioning, reduces sinus complications and postoperative symptoms, and allows a more buccal positioning of the implant head, thus facilitating prosthetic restoration. A new technique is currently being developed that involves placing extrasinus zygomatic implants by fixing them to the lateral sinus wall and the zygomatic bone. The authors

observed higher primary stability than with the classic technique since the implant is fixed to a larger amount of cortical bone [Figure 7].[17] The technique of tilting implants in the residual crestal bone of patients with maxillary atrophy allows placement of longer implants, thus increasing implant–bone contact area and implant primary stability; anchorage into the dense bone adjacent to the anterior sinus wall also contributes to increased stability. Posterior tilting of distal implants increases the distance between anterior and posterior implants, thus reducing the need for distal cantilevers; biomechanically, the distalization of the implant platform reduces the moments of force and improves the load distribution. Furthermore, tilted implants may suppress the need for bone grafting procedures in some cases, thus reducing biologic and economic costs and leading to higher patient acceptance.[18] Basal implantology also known as bicortical implantology or cortical implantology is a modern implantology system which utilizes the basal cortical portion of the jaw bones for retention of the dental implants which are uniquely designed to be accommodated in the basal cortical bone areas. The basal bone provides excellent quality cortical bone for retention of these unique and highly advanced Bone manipulation procedures in dental implants

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Use of subperiosteal implants

implants. The two types basal osseointegrated and basal cortical screw (BCS) basal implants are specifically designed to utilize strong cortical bone of the jaw. Screwable basal implants (BCS brand) have been developed with up to 12 mm thread diameter can be inserted into immediate extraction socket.[19] The subperiosteal implant was conceived out of the need to improve the retention and stability of full dentures in severely atrophied ridges. Today, its use is still limited to the severely atrophied ridge of either the mandible or the maxilla. However, the implant is used less commonly in the maxilla because it has a lower success rate there; in addition, qualities of the maxilla allow for more retentive and stable prostheses. Lower success rates in the maxilla can be attributed to the poorer quality of bone. Subperiosteal implants are more successful in the basal bone – rich mandible than in the maxilla, which is primarily cancellous bone.[20]

DISCUSSION Alveolar ridge modification is a prerequisite for both the implant and/or fixed prosthesis. It improves both the gingival and the bone architecture for esthetic and functional purposes. Peri-implant plastic surgery focuses on harmonizing peri-implant structures by means of hard tissue and soft tissue engineering. It includes bone structure enhancement, soft tissue enhancement, precision implant placement, and quality of prosthetic restoration. A deform ridge may result from teeth extractions, severe periodontal disease, abscess formations, etc., the deformity that exists in the ridge is directly related to the volume of the root structure and associated bone that is missing or has been destroyed.[21] When managing the edentulous maxilla, the clinician is often faced with a large pneumatized maxillary sinus and a very thin alveolar ridge in the anterior maxilla. Alveolar bone resorption can be accelerated with denture use, resulting in loss of vertical height and very thin bone separating the crest of the ridge from the large sinus and the floor of the nose. These patients could be treated in a conventional fashion with augmentation of the sinuses only. When this is done, the patient can be restored with implant-supported dentures or a fixed-detachable prosthesis, but the lost vertical dimension must be replaced with acrylic. To improve the implant-to-tooth ratio, vertical

augmentation is desired. If near ideal ridge height is obtained, the dentist and patient have several restorative options. The severely atrophic mandible is also challenging to restore. Placement of implants in the severely atrophic mandible can result in fracture, thus reconstruction with bone grafting is usually indicated. With larger bone stock, implants can sometimes be placed posterior to the mental foramina, allowing for restorative options other than placement of implants in the anterior mandible only. Bone grafts are widely used in the reconstruction of osseous defects in the oral and maxillofacial region. Autogenous bone grafts are generally obtained from the ilium, the rib, and the calvarium. These grafts can be easily obtained from these donor sites, but each site has associated morbidity. The maxilla and mandible are alternative sources of membranous bone and are thought to undergo less resorption than endochondral bone. A variety of local bone grafts, such as mandibular symphysis, mandibular body, mandibular ramus, and coronoid process, have been used in the oral and maxillofacial reconstruction. Intraoral bone donor sites are excellent alternatives for the augmentation of edentulous alveolar defects before implantation. GBR is a safe and effective technique for obtaining bone formation and placing dental implants in cases in which it would otherwise not be possible, even if an ideal membrane for treatment is not yet established. The technique of GBR, with nonresorbable membranes, is a very predictable technique and with excellent results, provided that you comply with the universally accepted surgical procedure, the surgeon should have extensive experience in handling, especially surgical soft tissue to cover the nonresorbable membrane, which is the key to success.[22]

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The bony expansion using osteotomes is a reliable and relatively noninvasive way of widening narrow ridges. The expansion of atrophic ridges by the osteotome technique does not require harvesting of bone, reduces operating time and postoperative morbidity, shortens rehabilitation time, and eliminates the risk of exposure of the membrane or bone graft that could lead to infection. In the same manner, ridge splitting technique is used to expand the edentulous ridge for implantation or insertion of interpositional bone graft. This technique is only suitable for enhancing ridge width. There must be adequate available bone height for implant placement, and no vertical bone defect should be present.[23] Alveolar distraction is a technique in constant evolution. A review of the literature within the past 14 years reveal that there are clear indications for its use, with outcomes similar to and sometimes even more predictable than traditional bone grafting techniques in preparation for implant placement. Although complications exist with alveolar distraction, it seems that most are minor and easy to manage. Appropriate patient selection and a better understanding of the technique are paramount in successful bone regeneration with alveolar distraction osteogenesis. Therefore, the success of the bone manipulation procedures relies on maintaining the integrity of the labial wall, which occurs as long as the periosteum remains intact. Since 80% of the blood supply is from the periosteum, we feel the high degree of the success in expanding very thin ridges is due to our ability to manipulate the thin cortical bone without disrupting the periosteal attachment to this bone.

**CONCLUSION** On the basis of available data, it is difficult or impossible to determine that one surgical procedure offers a better outcome than another, as far as predictability of the

augmentation and survival/success rates of implants placed in the augmented sites are concerned. Every surgical procedure presents advantages and disadvantages, which must be carefully evaluated before surgery. Moreover, it is not yet known if some surgical procedures that are widely used in clinical practice, such as sinus grafting procedures in the case of limited/moderate sinus pneumatization or reconstruction of atrophic edentulous mandibles with onlay autogenous bone grafts, are really useful for improving the long-term survival of implants. The predictable outcome of these procedures depends on several biologic principles that must be followed. Diagnosis, treatment planning, careful execution of the surgical treatment, postoperative follow-up, and appropriate implant loading are all important factors in achieving success.

### Grafting and Dental Implantation in Patients With Jawbone Cavitation: Case Series and 3-Year Follow-Up

Ya-Wei Chen, DDS,\* Miguel Simancas-Pallares, DMD,† Mauro Marincola, DDS,† and Sung-Kiang Chuang, DMD, MD, DMSc‡ Dental implantation is considered to be a predictable treatment modality for restoration of missing teeth. A satisfactory outcome should be achieved, if all of the following factors, including a comprehensive review of systemic and anatomical conditions, thorough treatment plans, and well-performed surgical procedures, have been taken into consideration. However, one of the intraoperative complications, displacement or migration of implants, may sometimes occur unpredictably. The most common situation is an accidental displacement of endosseous implants into maxillary sinus,<sup>1</sup> while such complications associated with mandible rarely happens. Nevertheless, some cases have been reported in which mandibular implants were found with loss of cortical bone engagement and displaced into marrow space of lower jawbones.<sup>2–5</sup> Primary stability is compromised as a result of implant overloading, insufficient bone quantity, or poor bone quality (eg, low density of trabecular bone, thinness of cortical bone, osteopenia, or osteoporosis).<sup>3,6</sup> Jawbone cavitation (BC) or osteocavitation is an anatomical phenomenal term, which has been used to describe the result of a disease process in which lack of blood supply to an area of bone resulted in a hole or hollowed out space in the jaw. Tracing back to history, this kind of jawbone lesion

was first described in the early 1870s by Noel. He depicted the noticeable defects in jawbone and considered them as a reason of dead or reduced vitality of bones.<sup>7</sup>

In 1915, Black<sup>8</sup> proposed possible causes and clinical findings of these cavitations. He found that although there were large destroyed areas within the jawbones, seldom did they cause pain, redness on the gingiva, swelling of the jaw, or rise in body temperature. Now various entities of diseases have been linked to

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**Purpose:** Jawbone cavitation (BC) is not uncommon and is considered to be related to some cases of unexpected implant displacement into deep jawbone space. Here, a series of cases with BC is described, in which the lesions were accidentally found and successfully treated by bone grafting and dental implantation. **Methods:** Thirty-four partially edentulous patients who were found to have BC during dental implant surgeries were included in this study. Alloplast bone substitute (β-tricalcium phosphate) grafting with immediate or staged locking-taper implant placement was performed. Bone filling and implants on BC were followed up to 36 months, and they were evaluated clinically and radiographically to verify treatment outcome.

**Results:** A total of 41 BCs were found at premolar and molar regions, which involved one or more teeth breadth. Nearly most of the lesions occurred in the mandible (95.1%, 39/41). Histologically, they were compatible with focal osteoporotic marrow defects. Fifty-two locking-taper implants and final restorations were delivered on 38 BCs. One implant failed due to loss of integration. The overall cumulative 3-year implant survival rate was 98.1%. **Conclusion:** By carefully examining and managing the surgical bed, the current treatment modality was shown to yield a satisfactory outcome for restoration of edentulous ridge with underneath BC. (Implant Dent 2017;26:158–164) **Key Words:** bone graft, cavitation, dental implant, jawbone, osteoporosis

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BC, such as osteonecrosis of the jaw (ONJ), neuralgia-induced cavitation osteonecrosis (NICO), condensing osteitis, focal osteoporotic marrow defect (FOMD), and traumatic bone cysts. There are still controversies on the etiology, diagnostic definition, and treatment of some of these disease entities. But an entity of disease which almost always causes pain mimicking the symptom of trigeminal neuralgia is mostly categorized to NICO,<sup>9,10</sup> whereas ONJ should have a clinical, radiographic, or pathological clue of bone necrosis.<sup>11,12</sup> If an implant has an unexpected displacement into a space which is not a true anatomical one, BC might be the cause. Although cases of BC were rarely reported in the literature and often regarded as clinically insignificant conditions, we have found a considerable number of cases during implant surgeries, which drew our attention. In this study, we present a consecutive 34 cases of BC which were found after surgical flap elevation and osteotomy for preparation of implant placement. Microscopic examination of the scarce tissue retrieved in the BC revealed FOMD as the diagnosis. Alloplast bone substitute (β-tricalcium phosphate [β-TCP]) grafting with immediate or staged dental implant placement was performed to reconstruct the jawbone defects and restore the missing teeth. This is the first study documenting an implant and graft treatment of BC. We emphasize the importance of careful examination of the surrounding tissue where the implants will be placed, and a concise literature review of FOMD is also presented. **MATERIALS AND METHODS** Between August 2012 and August 2014, 209

patients with edentulous ridge of maxillae or mandible were treated with at least one implant in the Dental Implant Unit of Oral Surgery and Medicine Department, University of Cartagena, Colombia. Medical charts were reviewed, and complete clinical and radiographic records of 34 consecutive cases with BC were retrieved for this study. All these patients with BC have been followed up for at least 3 years postoperatively. They were inquired for a complete medical and drug history about neoplasm, bone disease, metabolic disease, hematologic disease, and hormone therapy. Previous dental treatment history and the reason for extraction in the edentulous region were also collected. Approval for study conduction was obtained from the Ethical Board at the Dental Faculty Research Committee at the University of Cartagena (Ethics Review Committee, ERC approval No: Record No. 3, October 2014). All 34 patients came to the Dental Implant Unit, requesting for dental restorations on their edentulous region. None of them presented with symptoms of pain, swollen, inflammation, infection, paresthesia, or dysesthesia on

Fig. 1. Example of a case in which simultaneous bone grafting and implant placement was performed in 1 visit. A, Dental radiography of the edentulous region shows a 2- to 3-mm thick cortical bone. In the cancellous region, gross bone density seems normal, but some indistinct trabecular rarefaction can be noticed after careful inspection. B, After flap elevation and osteotomy made for surgical bed preparation, irregular surface on the inner wall of cortex is noted. C, The cavity wall was detected and explored by a periodontal curette, revealing a hollow space surrounding the site for dental implant. D, After thorough cleaning of the cavity by surgical curette and irrigation with normal saline, the cavity is filled with synthetic alloplast grafting material. E, The cavity should be completely filled with bone grafts and then distributed evenly, leaving only a space for implant placement. F, In this case, a short, 4.5 3 6-mm locking-taper implant is placed into the constructed hole and tapped 2 mm below the crest. G, The flap is closed for primary healing. H, Postoperative radiograph showed graft filling and implant in site. The implant shoulder is located 2 mm below the crest. (Note: The dental amalgam restoration on the second molar and composite resin restoration on the second premolar adjacent to the edentulous region were performed by the dentist in a local dental clinic. The quality was compromised. But because the patient refused to change the restorations due to no symptoms, we could only suggest the patient to keep follow-up at our clinic and have the restoration redone if any secondary caries or symptoms were found.)

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maxillofacial regions. There were totally 54 edentulous tooth positions needing to be restored. The teeth have been extracted for 21 to 420 days (mean:141695). All patients received thorough evaluation based on clinical dental conditions, and dental radiographs were taken for implant planning. Clinical examination included all routine preoperative implant surgery evaluations, eg, bone volume, jaw relations, intermaxillary distance, occlusal relation, and conditions of the opposing dentition. Anatomical conditions and possible pathological lesions in the jaws were evaluated with panoramic and intraoral periapical radiographs. Periapical radiographs were performed using radiovisiography with parallelism technique (Dr. Suni Plus; Suni Medical Imaging, San Jose del Oro, CA). There were unremarkable findings on clinical

examination in all patients. None of the radiographic examinations revealed obvious abnormal rarefaction shadows on their edentulous region (Fig. 1, A).

After fully explaining to the patients, they completed the written informed consent, and patients who had habits of cigarette smoking were requested to cease smoking at least 1 week before and for 3 months after surgeries.

Surgical and Restorative Treatment Antibiotics premedication was prescribed 1 day before the surgery, and all

the patients received 500 mg amoxicillin (or 300 mg clindamycin instead if the patient was reported to have allergic reaction to penicillin) every 8 hours for a total of 3 doses before surgeries. Immediately before surgical procedures, each patient was instructed to rinse the oral cavity with 0.2% chlorhexidine (Farpag Laboratories, Bogotá DC, Colombia) mouthwash for 1 minute and then received local anesthesia using lidocaine with adrenaline 1:100,000 (Xylocaine; Dentsply Pharmaceutical York, Pennsylvania). Surgeries began with a crestal incision made on the edentulous ridge, and full-thickness envelope or triangular flaps were reflected to expose the underlying bone. All of the cortical crestal surface of alveolar ridges were intact, thus osteotomies were performed until the planned diameter and depth had been reached (Fig. 1, B). There was no abnormal fluid discharge, but obvious loosening of bone density was felt when doing osteotomies in these cases. The walls and floor were carefully explored with a Lucas curette (Fig. 1, C). A hollow bone cavitation was found in the marrow space in all of our cases, and the extension of defects was determined using periodontal gauge probes.

There was nearly empty tissue but a little amount of blood in the bone cavity. Besides, sparse thin membranous-like tissue was found by curettage around the wall. Some of these obtainable tissues

were sent for histological examination to rule out any pathological conditions. Defects extending beyond the dental socket, with less than 5 to 6 mm width and 10 mm depth, were treated by simultaneous synthetic alloplast grafting (b-TCP; SynthoGraft, Bicon, MA) and locking-taper implant placement (Fig. 1, D–F). If the defect was more than the size mentioned above, only grafting was performed, and a 6-month clinical and radiographic follow-up was done for bone filling, and further dental implantation was arranged. A second stage of surgery was performed 4 to 6 months after implant placement with also a mid-crestal incision to uncover the submerged implants. A healing abutment was connected, and the wound was allowed to heal for at least 2 weeks. All the patients were then referred to one of the two prosthodontists for fixed implant restoration with either single crown or splinted bridge. There were totally 38 implants placed with simultaneous b-TCP grafting in 27 patients (32 BCs), while the other 7 patients (9 BCs) received b-TCP grafting for the first surgery and then staged dental implantation as the second surgery due to extensiveness of the BC. One experienced oral surgeon conducted all surgical treatments. After each stage of surgery, amoxicillin (500 mg) was prescribed every 8 hours for 7 days, and if the patient was allergic to penicillin or its derivatives, clindamycin (300 mg) every 8 hours was prescribed instead. All the patients were instructed to clean their teeth using finger-held gauze with essential oils (Listerine, Johnson & Johnson de Colombia, Bogota DC, Colombia). Follow-Ups All the 34 patients have been recalled for routine clinical examinations after grafting and implant surgeries, which were at 1, 2, and 4 weeks postoperatively. Sutures were removed at the 2-week follow-up. Then, they were recalled for the first postoperative radiographic examination 6 months after the first-stage procedure. Other radiographic

follow-up appointments were arranged 6 months after the second-stage procedure. All the patients have been followed up at least 6

Table 1. Patient Demographic (Age, Gender, and History of Diseases and Drugs) and Clinical Data (Numbers of BC and Implant-Related Information) Patient Characteristics (n = 34)  
 Age, mean  $\pm$  SD, y 55.44  $\pm$  11.87 Age range, y 25–83 Males/Females 11/23 Systemic disease (yes/no) 20/14 Skeletal problems (yes/no) 5/29 Hematological disorders (yes/no) 0/34 Taking drugs (yes/no) 24/10 Steroid therapy (yes/no) 2/32 Hormone therapy (yes/no) 0/34 Habit of cigarette smoking (yes/no) 2/32 Habit of alcohol consumption (yes/no) 9/25 Jawbone cavitation (n = 41) Position on maxillae/mandible 2/39 Dental implant characteristics (n = 52) Stage of dental implantation (1-time surgery/staged surgery) 38/14 Implants' position (anterior teeth/premolars/molars) 0/12/40 Implant length (5.0/6.0/8.0/11.0) 6/16/27/3

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months after first-stage operation, and the average follow-up duration was 24 months (range: 10–36 months).

Evaluation Method Implant-related parameters and any variables possibly related to jaw BC were retrieved. Descriptive statistics

(mean, ranges, and SD) were reported for the patients' systemic diseases, drug intakes, and previous dental histories (case of exodontia). Statistical analyses were performed using computer software (SPSS 17.0; SPSS Inc., Chicago, IL). Surgical complications were recorded according to the following:

Complications included symptoms of pain or paresthesia at surgical region, continuous objective inflammatory and infection signs, and persistent radiolucency or loss of graft on radiographic images. Implant failure is defined as (1) removal of implant due to any complication or (2) clinically

Fig. 2. The bar graph illustrating patients' dental treatment history at the region of BC.

Thirty patients with 45 missing teeth regions with BC underneath had RCTs history. Two patients (3 missing teeth regions with BC underneath) had tooth extraction due to chronic periodontitis and 1 patient (4 regions) due to tooth agenesis. One patient had 2 teeth extracted for orthodontic treatment need.

Fig. 3. Histological examination reveals hematopoietic and fatty marrow with a large area of dystrophic calcification and minimal cellular content (arrows). (hematoxylin and eosin stain, 340).

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mobile implant with the patient's awareness of loss of chewing function. The rate of complication or failure was expressed as a percentage among the total number of implants placed. Survival analysis and a life-table method were used to estimate the prognosis for the 6-month to 3-year cumulative survival rate of implants.

RESULTS Distribution of cases according to age, sex, medical and dental history, as well as BC and implant types is summarized in Table 1. The patients included 11 men and 23 women (aged 25–83 years), with a mean age of 55.44  $\pm$  11.87 years. Regarding medical history, 20 patients had systemic disease but not contraindicated local surgical treatments.

There are 7 patients (7/34) with hypertension and 4 patients (4/34) with diabetes mellitus, and all of them have been taking antihypertensive and antidiabetic agents. There is only one patient with osteopenia and another one with osteoporosis, and both of them were only taking calcium tablets. None of them had history of malignant neoplasms, hematological disorders, or autoimmune diseases. In regard to the drugs taken by the patients, most of them were prescriptions in correspondence to their systemic disease. Among them, 2 patients were under corticosteroid therapy for asthma, 2 patients were under insulin replacement therapy, and 1 patient was under thyroid replacement therapy. Besides, 3 patients took calcium tablets for dietary supplement, and over-the-counter nonsteroid anti-inflammatory drugs were taken occasionally. History of taking any bisphosphonate agents was denied. To analyze the possible related factor of BC, the reasons for extraction of teeth and dental treatment history in the region were also retrieved. Most of the patients with BC (30 of 34 patients with 45 missing teeth regions with BC underneath) had root canal treatments (RCTs) history, whereas the others had their teeth extracted due to chronic periodontitis (2 patients with 3 missing teeth regions) and tooth agenesis (1 patient with 4 missing teeth regions) and for orthodontic treatment needs (1 patient with 2 missing teeth regions) (Fig. 2). Of the 41 BCs found in 34 patients, nearly all (39/41) were found in the mandible and only 2 were in the maxillae. Seven patients were found to have BC in the mandible bilaterally. Twenty-seven patients (with 32 BCs) received b-TCP grafting and 38 dental implantation in one-time surgery, whereas the other 7 patients (with 9 BCs) received b-TCP grafting only and staged implant surgeries for 16 tooth positions were planned. After a 6-month follow-up examination, 6 patients showed bone fill in the BC and received dental implantation successfully (14 implants in 7 BCs). However, 1 patient had symptoms of throbbing pain 1 month postoperatively, and graft loss was noticed in his bilateral mandibular BC during follow-up period. Implant surgery was not performed. This patient had a medical history of osteoporosis, and it was found by the proximity of the BC to inferior alveolar nerves, which was considered to be the cause of pain.

**Histological Findings** Eight patients had available tissue from curettage for histological examination. All these multiple tissue slides showed adipose tissue with large dystrophic calcifications surrounded by hemorrhagic material and mixed inflammatory cells. No evidence of epithelial lining or malignant cells was noted (Fig. 3). The histopathological features, though nonspecific, resemble to that of the FOMD.

**Outcomes of Implant on BC** Of the 52 implants, 22 were of 5 and 6 mm length and 30 were of 8 and 11 mm. The implant diameter ranged from 4 to 6 mm. The implant positions were distributed in premolar (12/52) and molar (40/52) regions (Table 1). By the end of 3-year follow-up period, 1 implant failed and resulted in an overall failure rate of 1.9%. This failed implant was originally placed in the lower molar region with the graft as one-time surgery. A single crown has been restored on it. But loss of osseointegration at 25 months postoperatively was shown, and it was removed eventually. All the other remaining implants were free of complications and functioned well, resulting in a 3-year cumulative survival rate of 98.1% (Table 2).

**DISCUSSION** In this retrospective cohort study, we introduced an approach to treat BCs with synthetic alloplast grafting and dental implantation. All the patients had very similar clinical situations: They came without remarkable

symptoms and signs on the edentulous region to be treated by dental implantation, and hollow marrow space or bone cavitations were found not until osteotomies were made. We suspect that the BCs in these cases might all be FOMDs, for 8 of the cases with available specimen were histologically compatible with the diagnosis. FOMDs, with some synonyms such as focal osteoporotic bone marrow defects (FOBMDs) and osteoporotic bone marrow defects (OBMDs), are considered to be a rare condition of the jaws because they are asymptomatic and only some of the cases could be found on plain radiographic examination. The FOMD usually presents with a poorly demarcated radiolucency with variable density. Only when it is observed under radiography with other disease possibilities being considered, a biopsy will be performed and a pathological diagnosis be established.<sup>13</sup> Also because of that, the incidence of this jaw lesion is hard to determine.

Table 2. Life-Table Analysis and Cumulative Survival Curve of Implants on BCs Period, mo  
 Implants Failed Lost to Follow-Up CSR% 6 mo 52 0 0 100 6–12 mo 52 0 0 100 12–24 mo 48  
 0 0 100 24–36 mo 17 1 0 98.1

CSR indicates cumulative survival curve.

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However, a recent research analyzing 9723 cases of jawbone radiolucency showed that FOMD accounted less than 1% of the lesions.<sup>14</sup> The first available documented largest case series of FOMDs was in 1974,<sup>15</sup> which reported the detailed clinical, radiographic, and histological characteristics of a total of their own 197 cases. In addition to Barker's article, there were another 3 case series,<sup>16–18</sup> which showed remarkable consistency in their cases with regard to sex, age, symptoms, location etc. They constructed a typical epidemiological, clinical, radiographic, and histological description of FOMDs. After then, several individual case reports have demonstrated various FOMDs with different manifestations.<sup>3,13,19–22</sup> From all the above published literature, it can be summarized that FOMDs predominantly affected middle-aged female patients, and most cases occurred in the mandibular molar area. FOMDs can be located either in areas adjacent to teeth or of previous extractions.<sup>23</sup> Our cases, however, were all involved edentulous regions of jaws because the patients originally came for dental restorations and the BCs were found fortuitously. Recently, there were 2 case report articles of FOMDs being related to implant problems.<sup>3,21</sup> One of them was a report of 3 cases involving displacement of implants into the FOMDs immediately and 2 days after the implants were placed.<sup>3</sup> We would like to address the importance that such cases might not be uncommon, and some other reported cases with dental implants displacement into mandible marrow space might also be related to FOMDs,<sup>2,4</sup> and these aspects highlight the importance of an early detection through x-rays or even a higher resolution image examination such as computerized tomography. This can yield a more accurate treatment planning, and thus, a higher success rate can be guaranteed for the patient. The etiological factors of FOMDs are still unknown. Makek et al<sup>16</sup> reviewed 276 reported FOMD cases, and they found that 151 of them had concomitant pathological or odontogenic conditions. Shankland et al<sup>24</sup> however, summarized 3 theories proposed in the previous literature: (1) marrow hyperplasia after an increased demand for blood cells resulting in bone resorption, (2)

residual hematopoietic marrow persisting into adulthood, and (3) defective regeneration of bony trabeculae in an area of previous trauma, local inflammation, or surgery. They also provided a fourth theory that FOMD is an early form of ischemic osteoporosis secondary to a malfunction of blood flow within the marrow, based on half of their 100 FOMD cases showing evidence of degeneration, ischemia, or necrosis on microscopic examination. Because most of our cases had history of endodontic treatment on teeth being extracted, we are more prone to the theory of an abnormal post-healing process after extraction of the tooth predisposed to some inflammatory condition to the most possible cause of the lesions. We would also like to postulate that FOMDs might be a form of osteoporosis that solitarily or concomitantly occur with systemic osteoporosis because the peak population of FOMDs is quite the same with systemic osteoporosis which is very common among postmenopausal women. Current biomaterials used for endodontic irrigation during RCT, such as sodium hypochlorite (NaOCl), are reported to have deleterious effects on the bone structure. Some authors have reported localized interdental bone necrosis after RCT.<sup>25</sup> Specifically, Kerbl<sup>26</sup> described on dogs the degradation of the organic matrix, and grossly, NaOCl caused significant changes in cancellous structure, leaving it less dense and also large structural craters of apparent demineralization. Because FOMD's etiology is not fully understood and having in mind that in this study, most of the patients had previous RCT on those sites, it is possible that the material extrusion to the bone can explain the defect's presence. Moreover, further studies are needed to confirm these hypotheses. Nevertheless, the upmost strategy to deal with suspected BCs having an ill-defined radiolucent shadow is to consider other cystic, neoplastic, hematological, and also metastatic diseases as first differential diagnoses. Once aggressive or devastating lesions are carefully ruled out and the diagnosis of FOMD is made, in contrary to "watchful waiting," this article has provided a practical treatment protocol for them. Within a 3-year follow-up, a fair clinical outcome of grafting and dental implantation in these patients is shown. Although there was 1 case with a medical history of osteoporosis shown to have graft loss, no other complications were found. We thought a patient with a medical history of osteoporosis is another critical issue to draw attention, but we are not discussing it in this study.

**CONCLUSION** BC in the edentulous region is no more considered to be uncommon; it can result in implant displacement if overlooked. In this study, we consider that the BCs were corresponding to an entity of focal osteoporosis called FOMDs. They could be treated by allopast grafting and dental implantation, which is shown to have satisfactory clinical outcomes. Nonetheless, a long-term follow-up result of these cases and an in-depth exploration of the pathogenesis and correlation with local and systemic factors are still mandatory in future research.

**DISCLOSURE** The authors claim to have no financial interest, either directly or indirectly, in the products or information listed in the article.

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and patients' follow-up, and a third-year dental student, Salin Insignares, for his assistance in the data collection.

### 33. What can occur during an extraction?

- a. Overheating a tooth with a dental high-speed handpiece while cutting the crown to remove the tooth could create a "dry socket".
- b. A piece of calculus could be left behind in the socket that will result in a "cavitation" forming after the socket heals with bone.
- c. Raw honey could be used to heal a "dry socket".
- d. A and B are correct.
- e. All are correct.

Dry Socket Etiology, Diagnosis, and Clinical Treatment Techniques

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Abstract (J Korean Assoc Oral Maxillofac Surg 2018;44:52-58)

Dry socket, also termed fibrinolytic osteitis or alveolar osteitis, is a complication of tooth exodontia. A dry socket lesion is a post-extraction socket that exhibits exposed bone that is not covered by a blood clot or healing epithelium and exists inside or around the perimeter of the socket or alveolus for days after the extraction procedure. This article describes dry socket lesions; reviews the basic clinical techniques of treating different manifestations of dry socket lesions; and shows how microscope level loupe magnification of 6× to 8× or greater, combined with co-axial illumination or a dental operating microscope, facilitate more precise treatment of dry socket lesions. The author examines the scientific validity of the proposed causes of dry socket lesions (such as bacteria, inflammation, fibrinolysis, or traumatic extractions) and the scientific validity of different terminologies used to describe dry socket lesions. This article also presents an alternative model of what causes dry socket lesions, based on evidence from dental literature. Although the clinical techniques for treating dry socket lesions seem empirically correct, more evidence is required to determine the causes of dry socket lesions.

Key words: Alveolar, Dry socket, Fibrinolysis, Osteitis

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medicament<sup>1,3,4</sup>. (Fig. 2) The use of co-axial lighting and microscope-level magnification of 6× to 8× or greater facilitates the irrigation of a dry socket lesion and minimizes contact of the irrigation needle with exposed bone. Optimal visualization of the illuminated socket

ensures that the irrigant reaches all the internal aspects of the socket and removes all microscopic debris. The dry socket medicament should cover the exposed bone for several days with a resorbable, but durable cover, which will protect the bone from painful mechanical stimulation, food impaction, and bacterial infiltration<sup>1</sup>. The dentist might suture the lesion to retain the medicament or blood clot and create a dense suture barrier over the socket opening if it is determined that chronic food impaction prevents systematic socket healing. The dentist may also anesthetize the patient and try to induce bleeding into the socket by aggressively curetting the socket or using a round bur or No. 330 bur with copious irrigation to avoid over-heating the bone to drill several 1.0 mm deep holes in the socket bone while avoiding arteries, nerves, thin socket walls, or other vulnerable anatomical features. When treating a dry socket lesion, the objective is to optimize the lesion such that the socket is optimally capable of forming an enduring layer of epithelium that covers the exposed bone inside the socket and around the socket occlusal perimeter. A dry socket lesion may show exposed bone located superior to the projected location of the occlusal surface of the socket after the socket heals. This bone may be a protruding septum of bone or may be located on the socket occlusal perimeter. This superiorly-located exposed bone would be the last aspect of the socket to be covered by epithelium, since the bone, protruding superiorly to the projected occlusal surface.

socket lesions, and presents a comprehensive clinical approach to treating dry socket lesions, with an emphasis on how to achieve immediate coverage of exposed bone with such treatments. The author also presents a model of the causes of dry socket lesions based on current experimental knowledge. There is uncertainty in the dental literature about what causes dry socket lesions. Although some factors, such as smoking, oral contraceptive use, and presence of fibrinolytic activity in post-extraction sockets correlate with an increased incidence of dry socket, a definitive mechanism for explaining dry socket pathogenesis remains elusive<sup>1,2</sup>.

## II. Treatment of Different Manifestations of Dry Socket Lesions

A dry socket lesion can present such that the bone inside the socket is exposed, but there is no exposed bone on the socket occlusal perimeter, and all of the exposed bone is below the projected location of the occlusal surface of the socket when the socket eventually heals.(Fig. 1) The socket bone can be completely exposed or can be covered by food debris or weakly clumped bacterial material. There may be some healing, which is exhibited by narrowing of the socket occlusal diameter by epithelial growth. In this article, the basic treatment for dry sockets is to irrigate out food particles or bacterial material using chlorhexidine gluconate or saline and then fill the socket with a

Fig. 1. A dry socket lesion where the socket perimeter is fully covered with healing epithelium, but a septum of exposed bone is visible inside the socket. The occlusal aspect of the septum bone is inferior to the projected plane of the occlusal aspect of the socket when the socket fully heals. John Mamoun: Dry Socket Etiology, Diagnosis, and Clinical Treatment Techniques. J Korean Assoc Oral Maxillofac Surg 2018

Fig. 2. The dry socket lesion in Figure 1 after packing with an iodoform paste. John Mamoun: Dry Socket Etiology, Diagnosis, and Clinical Treatment Techniques. J Korean Assoc Oral Maxillofac Surg 2018

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thelium covers all the socket bone and cannot be irrigated away.(Fig. 4) When a previous dry socket becomes completely epithelialized, this demonstrates that the socket has overcome mechanical stimulation or bacteria that were inhibiting the healing process. From this point, the socket will systematically progress toward complete healing, and the dry socket complication phase of the post-extraction healing process is over. As a result, the dentist no longer needs to debride the socket or apply medicament. The occlusal surface of a healing dry socket may be concave and collect food particles or plaque. If irrigation of bacterial material or food particles reveals a healthy layer of epithelium underneath, the bacteria or food particles are not preventing epithelialization of the socket. Any discomfort can be managed with non-narcotic analgesics; strong narcotic analgesics are not required. A chlorhexidine gluconate mouth rinse helps disinfect the socket while healing continues. A patient presenting with a healing dry socket may state that the socket had been uncomfortable in the past few days (when the socket was in the dry socket stage), but now feels better and simply wants the dentist to check that the socket is healing. A dentist can use microscopes and co-axial illumination to verify that a previous dry socket lesion is fully covered by epithelium by probing the epithelium to determine the presence of tensile strength, indicating vital tissue, and that there is no exposed bone that elicits acute pain to probing.

### III. Proposed Causes of Dry Socket Lesions

Comprehensive reviews of the proposed causes of dry face of the healed socket, would be exposed to food particles or mechanical trauma that may erode epithelium growing over that bone. This bone, if mechanically stimulated, would be a source of acute pain until the end of the healing period. A dentist may anesthetize the patient and use a football diamond bur with copious irrigation to trim this bone to approximately 1 mm inferior to the projected occlusal surface of the healed extraction socket. Such trimming can result in the bone becoming immediately coverable by a blood clot or medicament, thereby reducing the total number of days that this hyper-sensitive bone is exposed and helping to ensure that epithelium will systematically grow over the remaining exposed bone of the dry socket. If the protruding bone is located on the socket occlusal perimeter, the dentist can reduce the bone to a level that is inferior to the occlusal aspect of the gingival tissue located just lateral to the protruding bone. If the gingiva on the socket occlusal perimeter is superior to all of the socket bone, a socket blood clot or dry socket medicament is more likely to cover the bone. For some dry socket lesions, the dentist may observe and trim bone that protrudes buccally beyond the projected surface of the healed socket.(Fig. 3) Microscopes, combined with head-mounted co-axial illumination, facilitate the visualization of the interface between the protruding bone and the gingiva lateral to the protruding bone and result in selective drilling of the bone and not the gingiva. A healing dry socket is a previous dry socket that is now completely covered with vital epithelium such that this epi

Fig. 3. A dry socket lesion with separate buccal and occlusal areas of exposed bone. John Mamoun: Dry Socket Etiology, Diagnosis, and Clinical Treatment Techniques. J Korean Assoc Oral Maxillofac Surg 2018

Fig. 4. Example of a previous dry socket lesion that is now fully covered with a layer of epithelium that does not wash away with irrigation. John Mamoun: Dry Socket Etiology, Diagnosis, and Clinical Treatment Techniques. J Korean Assoc Oral Maxillofac Surg 2018  
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causing a dry socket lesion<sup>6,9</sup>. However, although Birn found a correlation between the presence of fibrinolytic activity in extraction sockets and dry socket lesion pathogenesis, fibrinolysis may not be the cause dry socket lesions. Since fibrinolysis also increases capillary blood flow to the extraction socket, it might actually reduce the probability of dry socket lesion formation<sup>25-28</sup>. Dry socket lesions routinely exhibit an eventual stoppage of blood flow to the socket. This idiopathic ischemia counteracts the effect of fibrinolysis and is presumably a cause of dry socket lesion initiation and pathogenesis. As an alternative to Birn's fibrinolytic theory, the author proposes a different model of dry socket lesion initiation and pathogenesis. In a high-stress extraction, that puts high compressive forces on alveolar bone surrounding the tooth, events are initiated that will cause, over a 24- to 96-hour period following the extraction, the necrosis of osteoblasts lining the intaglio surface of the socket. The necrosis of the osteoblasts may initiate fibrinolytic activity that lyses any blood clot that may have formed after the extraction, or the blood clot may dislodge because the necrotic osteoblasts lose the ability to metabolically integrate with the blood clot. Also, approximately at the time of osteoblast necrosis, the socket stops bleeding, even though the fibrinolytic activity should theoretically cause increased bleeding to the extraction socket to bring immune cells and complements to the socket to begin resorbing the necrotic osteoblasts. This idiopathic socket ischemia event may prevent an initial blood clot to reform through additional bleeding and may prevent the immune system from accessing the site through local capillaries to initiate an inflammatory response to resorb the necrotic bone cells. The necrotic bone cells are then exposed and uncovered for several days, resulting in the major symptom (or morbidity) of dry socket lesions, acute pain of the exposed socket to mechanical stimulation that lingers for several days until the bone becomes completely covered by healing epithelium. During a traumatic extraction, heavy luxation or forceps forces transfer to the jawbone surrounding the roots and may crush bone on the intaglio surface of the extraction socket<sup>1,10,29</sup>. This can induce necrosis or apoptosis of osteoblasts within the extraction socket<sup>30-32</sup>. Studies have shown that mechanical stress (excess tensile or compression forces) on osteoblasts can activate cellular signaling pathways that lead to osteoblast apoptosis<sup>30-33</sup>. Also, the percentage of apoptotic osteoblasts increases over 24 hours after the initial compressive force application<sup>30</sup> and increases in proportion to the compressive force<sup>30,33</sup>. The necrosis of bone cells, occurring over a >24-hour delay socket lesions and of the factors that correlate with increased dry socket incidence can be found in the literature<sup>1,2,5-9</sup>. One hypothesis is that bacteria initiate dry socket lesions or prolong their duration<sup>1,2,5-10</sup>. However, there is little evidence that antibiotics given after an extraction reduce dry socket incidence<sup>11-13</sup>. An antiseptic Chlorhexidine gel, placed prophylactically in extraction sockets after the procedure, does not significantly reduce dry socket incidence<sup>14,15</sup>. However, one meta-analysis found that systemic antibiotics given before third molar surgery reduced dry socket incidence<sup>16</sup>. Overall, these findings suggest that reducing bacterial counts around extraction sockets may only result in an insignificant reduction in dry socket incidence.

#### IV. Proposed Model of Dry Socket Lesion Pathogenesis

A model of dry socket lesion pathogenesis can explain various facts about dry sockets including the findings that smoking<sup>2,17,18</sup> and use of oral contraceptives<sup>2,18</sup> increase the incidence of dry socket lesions. In addition, the model can also demonstrate that there can

be a 24- to 96-hour delay after an extraction before dry socket lesions appear<sup>2,5</sup>; that traumatic extractions, where heavy luxation or forceps forces are required to extract teeth particles, increase the incidence of dry socket lesions<sup>19</sup>; that plasmin-induced fibrinolysis activity seems higher in dry socket lesions compared to nondry-socket post-extraction sockets<sup>2,6,9</sup>; and that bacteria do not seem to initiate dry socket lesions<sup>11-13</sup>. Such a model should explain whether or not inflammation causes dry socket lesions. Birn observed high concentrations of plasmin and increased fibrinolytic activity in the alveolar bone lining dry socket lesions<sup>6,9</sup>. Plasminogen, the precursor of plasmin, circulates in the blood and binds to clots at wound sites. Various tissue activators, including tissue-type and urokinase-type plasminogen activators<sup>20,21</sup>, convert plasminogen to plasmin<sup>6,20-22</sup>. Plasmin is experimentally identified as an important molecule for inducing inflammation<sup>20,22-24</sup> because it has been found to induce fibrinolysis to dissolve blood vessel clots, increase local capillary permeability, and attract inflammatory cells and its complements to wound sites. Birn hypothesized that trauma during an extraction or the presence of a bacterial infection somehow facilitates the release of plasminogen tissue activators in the post-extraction socket, resulting in the plasmin induction of fibrinolysis that dislodges the blood clot that formed after the extraction and

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Due to the lack of blood flow to the intaglio surface of the socket, the immune system cells and their complement factors cannot be brought to the intaglio surface of the socket to resorb the necrotic bone cells lining the socket. Instead, clinical observation seems to show that the socket heals by a mechanism where vital epithelium, initially present at the outer perimeter of the socket, grows gradually from the outer perimeter of the socket inferiorly into the socket down to the apex of the socket. As the vital epithelium gradually covers the surface area of the socket intaglio surface, the epithelium brings blood vessels, immune system cells, and their complements in direct contact with the necrotic bone cells of the socket to begin resorbing the necrotic bone cells. This process of epithelium growth may take several days; during this time, the uncovered bone is painful to the touch and is vulnerable to painful contact with bacterial biofilm or food impaction. This model of dry socket pathogenesis and healing implies that inflammation does not fundamentally cause dry socket lesions and is not the cause of dry socket morbidity (Fig. 5) because ischemia will prevent an inflammatory event from occurring at the dry socket lesion site. Therefore, this model questions the use of terminology such as “alveolar osteitis,” or “fibrinolytic osteitis,” or any other term using the inflammation suffix “-itis” to describe dry socket lesions. Instead, the author suggests an alternative terminology for the dry socket phenomenon: “post-extraction peri-alveolar exposed bone ostealgia syndrome.”

#### V. Evidence for the Model of Dry Socket Lesion Pathogenesis

There is evidence that reduced post-extraction socket blood flow facilitates dry socket lesion formation. Smoking<sup>17,18</sup> and use of oral contraceptives<sup>18</sup> both facilitate blood clotting throughout the body<sup>35</sup> and may reduce blood circulation into the extraction socket. Both smoking and use of oral contraceptives correlate with an increased incidence of dry socket lesions<sup>2</sup>. Traumatic extractions correlate with dry socket lesion incidence<sup>19</sup>. The incidence of dry socket lesions is lower for non-surgical extractions (that do not require tooth sectioning) compared to surgical extractions<sup>15,18,36,37</sup>. This may be due to a correlation between the need to section a tooth and the need for heavy luxation forces to

remove a tooth or individual roots. The highest rate of dry socket incidence among all teeth types occurs with the extraction of mandibular third molars.

period after an extraction, may result in the bone cells releasing urokinase plasminogen tissue activator, which is the main plasminogen activator released in dry socket lesions<sup>21</sup>. The urokinase plasminogen tissue activator then converts plasminogen to plasmin. The plasmin may directly result in the lysis of a blood clot that initially formed in the socket. However, a major function of plasmin is to initiate blood vessel perfusion to bring blood, immune system cells, and complements to the intaglio surface of the socket to begin resorbing the necrotic osteoblasts. In dry socket lesions, however, an idiopathic blood vessel ischemia event is eventually observed that prematurely blocks this capillary perfusion-mediated immune system activation process. The cause of ischemia at a dry socket lesion site is unknown. Theoretically, the high forces of the extraction may crush and occlude blood vessels within the bone forming the intaglio surface of the socket (although there is no experimental evidence for or against compression-induced blood vessel occlusion existing in dry socket lesions). Some socket bone may be dense, with few blood vessels per unit of socket area, or a socket may be observed to only bleed from the apical aspect, making these sockets intrinsically incapable of significant bleeding. Smoking or oral contraceptive use may also reduce systemic blood circulation<sup>17,18</sup>. In addition, the pro-bleeding effect of plasminolysis may be counteracted chemically by pro-ischemia thrombin activity<sup>34</sup> at the dry socket wound site.

Fig. 5. Example of a maxillary posterior dry socket lesion surrounded by a viral outbreak. Although the outbreak may theoretically increase generalized inflammation around the dry socket, it is unknown if the outbreak increases pain or the duration of the dry socket or is only coincident with the lesion. John Mamoun: Dry Socket Etiology, Diagnosis, and Clinical Treatment Techniques. J Korean Assoc Oral Maxillofac Surg 2018

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heavy luxation or forceps forces on the tooth. However, the sectioned individual roots still require heavy luxation forces to extract them. This often occurs when extracting endodontically treated roots that may be partially or fully ankylosed within the surrounding alveolar bone. ● Teeth with ellipsoid cross sections (particularly maxillary canines and two-rooted maxillary premolars) often cannot be extracted by rotating in a superior direction inside the socket using forceps, unless heavy forces are used. A root may be difficult to extract if it has an hour-glass cross-sectional shape due to mesial and distal concavities or if the root is ankylosed due to endodontic treatment. The dentist may be able to extract ellipsoid roots with minimal stress on the surrounding alveolar bone by sectioning the coronal 2/3 of the root mid-way between the buccal and lingual aspects of the root or by removing bone that has grown into the mesial and distal root concavities of the root to create circular cross sections of the sectioned teeth fragments.

## VII. Conclusion

This article described different manifestations of dry socket lesions, summarized the treatment approaches for each different manifestation, reviewed the proposed causes of dry socket lesions, described and presented a model of dry socket lesion pathogenesis, and proposed a different terminology for the dry socket phenomenon. More evidence is needed to prove the scientific validity of techniques of dry socket lesion treatment, to validate the proposed model, and to determine which factors cause dry socket lesions.

### Author's Contributions

J.M. developed the article concept, took clinical photographs, performed background research and wrote the manuscript.

### Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Mandibular third molars are often deeply embedded in dense bone and have the highest incidence of root dilacerations among teeth<sup>38-40</sup>. Mandibular third molars may have roots that are not radially co-axial with the imaginary radial axis on which the dentist places luxation forces to remove the root, especially if difficult access limits the number of possible ways of positioning luxation instruments. These factors may obligate a dentist to use heavy forceps or luxation forces, even after root sectioning, to extract mandibular third molars, and these heavy forces may transmit to the surrounding jawbone. Crawford<sup>41</sup> first described dry socket lesions, using a case report where he extracted a mandibular third molar "with great difficulty," and may not have sectioned the tooth, given the limited technologies in 1896. The incidence of dry socket lesion formation is lower with maxillary third molar extractions compared to mandibular third molar extractions. Maxillary third molars often have conical roots embedded in cancellous bone bounded by thin buccal bone, requiring less force for removal. Extracting teeth that are in cancellous bone may result in multiple sharp points of cancellous bone severing multiple blood vessels, which may ensure bleeding into the post-extraction socket and blood clot formation.

### VI. Scenarios That Result in High Stress Extractions

One example of a low stress extraction is if the dentist sections teeth before attempting extraction using heavy luxation and forceps forces. Also, infected teeth where the periodontal ligament has been resorbed by an underlying abscess can often be extracted with minimal stresses on surrounding alveolar bone, even if the roots feature ellipsoid cross sections. However, various situations can result in a tooth extraction where heavy stresses are placed on the surrounding jawbone: ● A dentist may extract a multi-rooted tooth using heavy luxation and forceps forces, moving the tooth back and forth to expand the socket to facilitate tooth extraction without sectioning the tooth roots that may be interlocked in bone. ● A dentist may initially attempt to extract a multi-rooted tooth using heavy luxation and forceps forces, but after placing heavy forces on the tooth, decides to section the tooth<sup>39</sup>. Sectioning the tooth results in less force needed to extract the tooth, but the heavy forces placed on the tooth prior to sectioning stressed the jawbone. ● A dentist sections a multi-rooted tooth prior to placing

Honey a sweet approach to alveolar osteitis: A study Vibha Singh, U. S. Pal, Ranjana Singh,<sup>1</sup> and Nikita Soni Department of Oral and Maxillofacial Surgery, KGMU, Lucknow, Uttar Pradesh, India <sup>1</sup>Department of Biochemistry, KGMU, Lucknow, Uttar Pradesh, India Address for correspondence: Dr. Vibha Singh, A-43 Krishna Nagar, Lucknow, Uttar Pradesh, India. E-mail: moc.liamg@ihnavuhgarhgnisabhiv Copyright : © National Journal of Maxillofacial Surgery This is an open-access article distributed under the terms of the Creative Commons Attribution Noncommercial-Share Alike 3.0 Unported, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Abstract Background:** Honey is one of the oldest known medicines. Its use has been rediscovered in later times by the medical profession, especially for dressing wounds. It has been reported from various clinical studies on the usages of honey as a dressing for infected wounds that the wound become sterile in 3-6 days, others have also reported that the honey is effective in cleaning up infected wound. **Materials and Methods:** The 54 patients of dry socket were selected from the from the outpatient department of oral and maxillofacial surgery. A diagnosis of dry socket was made clinically. This study was under taken to evaluate the effect of honey dressing in management of dry socket. **Results:** In this study there was significant reduction if inflammation, hyperemia, edema and exudation after honey dressing that results in soothing effect and reduction in pain and discomfort. There was also significant reduction in CRP level post operative days. There was side effect of honey was observed in our study, so it can be used as alternative for the management of dry socket. **Discussion:** It has also been reported that the honey dressing halt advancing necrosis. It has also been found to act as barrier preventing wounds from becoming infected, preventing cross infection, and allowing burn wound to heal rapidly. **Conclusions:** There are no side effects of honey. Excess use of eugenol, can lead to necrosis of bone. The honey can be used as medicament for the management of dry socket. **Keywords:** Dry socket, eugenol, honey

**INTRODUCTION** Honey is one of the oldest known medicines and its use has been rediscovered in later times by the medical profession, especially for dressing wounds. It has been reported from various clinical studies on the usages of honey as a dressing for infected wounds that the wound become sterile in 3-6 days, others have also reported that the honey is effective in cleaning up infected wound. It has also been reported that honey dressing halt advancing necrosis. Hence the honey can be used for the management of dry socket. In Ayurveda, - honey is considered to positively affect all three primitive imbalance of the body. Charak and Sushruta applied honey dressing for sores. In the third century, Greek philosopher Celsus used mixes honey and bran to treat burn. Dry socket or alveolar osteitis is one of the most common post-operative complications following the extraction of permanent teeth. It is a condition in which there is a loss of clot from the socket clinically post-operative discomfort can range from simple local inflammation to classic alveolar osteitis including halitosis regional trismus dull throbbing pain irradiating from empty socket, normally to ipsilateral ear, temporal region or eye. Occasionally regional lymphadenopathy is also noted. It is believed to be multi factorial in origin and these are some commonly etiological aggravating and precipitating factors. Oral microorganism, trauma during surgery, roots and bone fragments remaining in the cavity, excessive curettage and irrigation, dislodgement of blood clot, oral contraceptives and smoking are the some of the important factors.

There are various treatment modalities used for management of dry socket like bland obtundant dressing, pain reducing dressing such as zinc oxide euginol dressing, anti-infective agents, systemic or local, antifibrinolytic agent, surgical intervention to remove necrotic clot and encourage the formation of blood clot. METHODOLOGY

A total of 54 patients of dry socket were selected from the out-patient Department of Oral and Maxillofacial Surgery. The informed consent was taken prior to treatment. Patients with systemic illness like diabetes, pregnant and lactating female were not included in the study. Sterile gouge soaked with honey was used as a dressing. A diagnosis of dry socket was made clinically. This packing is changed until the post-operative pain symptoms subsided. This study was under taken to evaluate the effect of honey dressing in management of dry socket [Tables [Tables11–6].

All the 54 patients had pain and necrotic slough and 18 patients had halitosis.

In 41 patients symptoms developed at 3rd day and 9 patients reported at 4th day after extraction.

Symptoms-resolutions of symptoms in 30 patients all the symptoms subsided at 5th day in other 16, patient on 6th day and in 3 patients on 8th day after extraction [Figures [Figures11–3].

C-reactive protein (CRP) reduced significantly at post-operative.

The CRP levels are often elevated in patients with odontogenic infection and post-operative complications.[1] Rapid reductions in serum CRP level indicate successful treatment. It is a cytokine induced acute phase protein that increases in concentration as a result of inflammation. In our study, CRP reduced significantly at post-operative.

No adverse effect have been reported in the present study, honey has been used topically on wound over thousands of years without any adverse effect.

Allergy to honey is rare but there could be an allergic reaction to either the pollen or the bee protein in the honey. Honey gives a fast rate of tissue regeneration and suppression of inflammation, edema exudation and malodor in the wounds.

Honey can be expected to have a direct nutrient effect on regenerating tissues because it contains a wide range of amino acids, vitamins and trace elements in addition to large quantities of readily assimilable sugars. DISCUSSION Natural products have been used for several years in folk medicine. Honey has an effective antibacterial potential to combat oral pathogens and hold promises for the treatment of periodontal diseases and mouth ulcers. Honey was used to treat infected wounds as long ago as 2000 years before bacteria was discovered to be cause of infection. 50 AD Dioscorides described honey as being good for all rotten and hollow ulcers. Honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes. Gram-positive and Gram negative microorganisms.[2]

The antibacterial property of honey was first recognized in 1892 by van Ketel. The minimum inhibitory concentration was found to a range from 1.8% to 10. 8% (v/v) indicating that the honey has sufficient antibacterial potency to stop bacterial growth if diluted at least 9 times due to its hygroscopic properties, its acidic pH and hydrogen peroxide.[2]

Glucose + H<sub>2</sub>O + O<sub>2</sub> → Gluconic acid + H<sub>2</sub>O<sub>2</sub>. It serve to preserve the honey. The major antibacterial activity in honey has been found to be due to hydrogen peroxide produced enzymatically in the honey. Phytochemical factors it has enzyme and tissue nutrition

material and vitamins that help repair tissue directly. The proliferation of peripheral blood B lymphocytes and T lymphocytes in cell culture is stimulated by honey at concentration as low as 0.1%. It also stimulates monocytes in cell culture to release cytokines tumor necrosis factors- $\alpha$ , interleukin1 (IL-1) and IL-6 which activate the immune response to infection.[2]

There are some other explanations of the antibacterial activity of the honey like. Osmotic effect The honey is saturated or supersaturated solution of the sugar 84% being the mixture of fructose or sucrose the water content is usually 15-21% by weight. The strong interaction of these molecules with water molecules leaves very few of the water molecules available for microorganisms. This free water is what is measured as water activity (aw) : m0 can value for the honey have been reported from 0.562 to 0.62. Many species of bacteria have their growth completely inhibited if (aw) is in the range of 0.94-0.99. Acidity - it is quite acidic its pH is from 3.2 to 4.5, low enough to be inhibitory for many pathogens. Hydrogen peroxide The major antibacterial activity in honey has been found to be due to hydrogen peroxide enzymatically in the honey. The glucose oxidase enzyme is secreted from the hypopharyngeal gland of the bee into the nectar to assist in the formation of honey from the nectar.

$\text{Glucose} + \text{H}_2\text{O}_2 + \text{O}_2 \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2$ . This serves to preserve the honey. The hydrogen peroxide produced would be the effect as a sterilizing agent only during ripening of the honey. Full strength honey has a negligible level of hydrogen peroxide because this substance is short lived in the presence of transition metal ions and ascorbic acid in the honey which catalyzes its decomposition to water and oxygen. On dilution of honey the activity increases by a factor of 2500-50,000 thus giving a slow release antiseptic at a level which is antibacterial but not tissue damaging.[2,3] Phytochemical factors All the antibacterial activity does not account for peroxide generating system it shows that there must be an additional antibacterial factor involved. Several chemicals with antibacterial activity has been identified in the honey by various researches, pinocembrin, terpenes, benzyl alcohol, 3,5-dimethoxy-4-hydroxy benzoic acid, methyl 3,5-dimethoxy 4-hydroxy benzoate, 3,4,5 trimethoxy benzoic acid, 2 hydroxy, 3 phenyl propionic acid, 2 hydrobenzoic acid and 1,4 dihydroxy benzene.

It appears that the honey from certain plants has better antibacterial properties than from others but there are no sufficient evidence.[3] It has been reported from various clinical studies on the uses of honey as dressing for the infected wounds that the wounds become sterile in 3-6 days and other reported that honey is effective in cleaning up infective wounds. It has also been reported that the honey dressing halt advancing necrosis. It has also been found to act as a barrier preventing wounds from becoming infected, preventing cross infection and allowing burn wound to heal rapidly. Honey has been reported to promote the formation of clean healthy granulation tissues. It also reduces inflammation, reduced hyperemia, edema, exudation and soothing effect when applied to wounds.[2,3] Hence its physical properties provide a protective barrier, by osmosis, create moist healing environment, in the form of the solution, not to stick underlying tissue. The antibacterial properties of honey prevents bacterial colonization, of the moist environment and no impairment of the healing process through adverse effect on wound tissue to the contrary it appears to have a stimulatory effect on tissue regeneration.[4,5,6] There are clear indication of anti-inflammatory action, so this can be used as a therapeutic agent for the dry socket. CONCLUSIONS There are no side-effects of honey. Excess use of eugenol, can

lead to necrosis of bone. The honey can be used as a medicament for the management of dry socket.

### 34. What statement is incorrect about “cavitations”?

- The medical terms for a dental cavitation are “cavitation osteonecrosis” or “ischemic osteonecrosis”.
- “Cavitations” may not show obvious symptoms, but they could result in chronic inflammation and infection manifesting in other areas of the body.
- A Cone Beam CT Scan is all that is necessary to prove a “cavitation” is present.
- Some “cavitations” can cause pain and are called “Neuralgia-Inducing Cavitation Osteonecrosis” (NICO).

### Abstract

#### OBJECTIVE:

We hypothesized that, similar to idiopathic hip osteonecrosis, the T-786C mutation of the endothelial nitric oxide synthase (eNOS) gene affecting nitric oxide (NO) production was associated with neuralgia-inducing cavitation osteonecrosis of the jaws (NICO).

#### DESIGN:

In 22 NICO patients, not having taken bisphosphonates, mutations affecting NO production (eNOS T-786C, stromelysin 5A6A) were measured by polymerase chain reaction. Two healthy normal control subjects were matched per case by race and gender.

#### RESULTS:

Homozygosity for the mutant eNOS allele (TT) was present in 6 out of 22 patients (27%) with NICO compared with 0 out of 44 (0%) race and gender-matched control subjects; heterozygosity (TC) was present in 8 patients (36%) versus 15 control subjects (34%); and the wild-type normal genotype (CC) was present in 9 patients (36%) versus 29 controls (66%) ( $P = .0008$ ). The mutant eNOS T-786C allele was more common in cases (20 out of 44 [45%]) than in control subjects (15 out of 88 [17%]) ( $P = .0005$ ). The distribution of the stromelysin 5A6A genotype in cases did not differ from control subjects ( $P = .13$ ).

#### CONCLUSIONS:

The eNOS T-786C polymorphism affecting NO production is associated with NICO, may contribute to the pathogenesis of NICO, and may open therapeutic medical approaches to treatment of NICO through provision of L-arginine, the amino-acid precursor of NO.

Neuralgia-inducing cavitation osteonecrosis in a patient seeking dental implants Yazad R. Gandhi, U. S. Pal,<sup>1</sup> and Nimisha Singh<sup>1</sup> Department of Oral and Maxillofacial Surgery, Saifee Hospital, Mumbai, India <sup>1</sup>Department of Oral and Maxillofacial Surgery, K G Medical University, Lucknow, India Address for correspondence: Dr. Yazad R. Gandhi, Aderbad Apartments, 1st Floor, 34, Hughes Road, Babulnath, Mumbai – 400 007, India. E-mail: ni.oc.eraclatnedetelpmoc@ofni Copyright : © National Journal of Maxillofacial Surgery This is an open-access article distributed under the terms of the Creative Commons Attribution Noncommercial-Share Alike 3.0 Unported, which permits unrestricted use,

distribution, and reproduction in any medium, provided the original work is properly cited. Abstract NICO (Neuralgia-Inducing Cavitation Osteonecrosis) is one of the jawbone versions of ischemic osteonecrosis, a common disease affecting any bone but with special affinity for those of the hips, knees and face. By definition, NICO is associated with pain. Osteonecrosis itself may or may not be painful. It may or may not affect multiple sites. It is a problem of poor blood flow through the marrow. Patients can trace the onset of their pain subsequent to one or more extractions, perhaps decades ago. Notably, if patients had infections following their extractions or even dry sockets, there was a greater likelihood of NICO development. NICO's can refer pain across the midline; that is, a lesion in the right jaw can cause pain on the left side of the face, head, neck or body. Yes, NICO's can refer pain to various areas of the body, including the neck, arms and hands, legs and feet, groin.

Keywords: Avascular, cavitation, neuralgia, osteonecrosis INTRODUCTION History and overview

Most people know what we mean when we say cavity, but the word cavitation is confusing. A cavity is a hole in the tooth, whereas a cavitation is a hole in bone. Unlike most tooth cavities, bone cavitations cannot be detected by simply looking at the bone, and even using X-rays, many cavitations are missed. The term cavitation was coined in 1930 by an orthopedic researcher to describe a disease process in which a lack of blood flow into the area produced a hole in the jawbone and other bones in the body. Dr. G. V. Black, the father of modern dentistry, described this cavitation process as early as 1915 when he described a progressive disease process in the jawbone, which killed bone cells and produced a large cavitation area or areas within the jawbones. He was intrigued by the unique ability of this disease to produce extensive jawbone destruction without causing redness in the gingiva, jaw swelling, or an elevation in the patient's body temperature. Essentially, this disease process is actually a progressive impairment which produces small blockages (infarctions) of the tiny blood vessels in the jaw bones, thus resulting in osteonecrosis or areas of dead bone. These dead cavitation areas are now called neuralgia-inducing cavitation osteonecrosis (NICO) lesions.[1] In his book on oral pathology, Dr. Black suggested surgical removal of these dead bone areas.

The cause of NICO is allegedly avascular osteonecrosis (AO) (also known as ischemic osteonecrosis). This bone ischemia would result from a chronic low-grade infection or susceptibility to thrombosis.

The suggested treatment for NICO involves surgical decortication and debridement of bone via curettage.[2] CASE REPORT A female patient, 56 years of age, reported to the maxillofacial outpatient department with a chief complaint of pain in the lower jaw since the last 2 years. She had consulted numerous dental surgeons who provided a conservative approach comprising medication and endodontic treatment of neighboring teeth.

In addition to this, she had received treatment by an oral surgeon for trigeminal neuralgia (Carbamazepine 200 mg TID). There was no relief from the said treatment; so, the patient was referred to a neurosurgeon who put her on a regimen of Gabapentin.[3]

After 2 weeks of the same, the patient still complained of the same symptoms. On examination, the orthopantomogram (OPG) [Figure 1] radiograph revealed some rarefaction of trabecular pattern in the molar region on the left side of the mandible. The area was opened under local anesthesia by way of a crestal incision with vertical releasing limbs. The cortical bone in the alveolar region was discolored with a darkish hue. Upon probing, the bone was soft and demonstrated a drop-in effect with a sharp instrument.

The whole of the affected bone was removed using rotary instruments and the area debrided [Figure 2]. A bi-layered collagen membrane was used to cover the defect and primary closure achieved using 4-0 vicryl sutures.

The patient was reviewed 3 days later and was found to be doing well with almost complete resolution of the pain, the only discomfort being that from the surgery and sutures.

After a week, the patient reported for review and near-complete resolution of earlier symptoms was noted.

The patient reported 3 months later with complete resolution of symptoms and a repeat OPG radiograph showed good radiodensity in the area [Figures [Figures 33 and 44].

Three endosseous implants were planned in the region for rehabilitation of both the premolars and molars [Figure 5]. **CONCLUSION** Because of the lack of clear etiological data, a NICO diagnosis should be considered only as a last resort when all possible local odontogenic causes for facial pain have been eliminated. It is recommended that NICO be included in the differential diagnosis of idiopathic facial pain syndromes.

If a NICO lesion is suspected in relation to an endodontically treated tooth, if possible, periradicular surgery and curettage should be attempted, not extraction. In addition, the practice of recommending the extraction of endodontically treated teeth for the prevention of NICO, or any other disease, should be avoided.

Peripheral Neuropathic Facial/Trigeminal Pain and RANTES/CCL5 in Jawbone Cavitation  
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**Introduction.** In this study, we elucidate the possible causative role of chronic subclinical inflammation in jawbone of patients with atypical facial pain (AFP) and trigeminal neuralgia (TRN) in the local overexpression of the chemokine regulated on activation and normal T-cell expressed and secreted (RANTES/C-C motif ligand 5 CCL5). Neurons contain opioid receptors that transmit antipain reactions in the peripheral and central nervous system. Proinflammatory chemokines like RANTES/CCL5 desensitize  $\mu$ -opioid receptors in the periphery sensory neurons and it has been suggested that RANTES modifies the nociceptive reaction.

**Materials and Methods.** In 15 patients with AFP/TRN, we examined fatty degenerated jawbone (FDOJ) samples for the expression of seven cytokines by multiplex analysis and compared these results with healthy jawbones. Results. Each of these medullary jawbone

samples exhibited RANTES as the only highly overexpressed cytokine. The FDOJ cohort with AFP/TRN showed a mean 30-fold overexpression of RANTES compared to healthy jawbones.

**Conclusions.** To the best of our knowledge, no other research has identified RANTES

overexpression in silent inflamed jawbones as a possible cause for AFP/TRN. Thus, we hypothesize that the surgical clearing of FDOJ might diminish RANTES signaling pathways in neurons and contribute to resolving chronic neurological pain in AFP/TRN patients.

1. Introduction The etiology of chronic facial pain is challenging to diagnose and difficult or frustrating to treat. Many different concepts have been presented and discussed, for example, the presence of a neuroma, implying that the nerve has been damaged in the periphery, and intracranial vascular compression of the trigeminal nerve root at the base of the skull. In 1997, Jannetta published a long-term follow-up study of the surgical approach to move the superior cerebellar artery away from the nerve root, maintaining the artery in its new position with a suture [1]. Various complementary various medical treatments for this problem, such as use of carbamazepine, have been reported [2]. Chronic facial pain can also be related to the temporomandibular joint (TMJ) and can be due to involvement of the cervical plexus [3]. Different terms have been used to describe atypical facial pain (AFP) such as atypical odontalgia (AO, also known phantom tooth pain), psychogenic toothache, and persistent dentoalveolar pain disorder [4]. International associations for the study of pain have adopted the term “persistent idiopathic facial pain” (PIFP) to replace AFP [5]. Pain is also one of the hallmarks of inflammation. Acute trigeminal pain is unavoidable given our interaction with dental decay, but it is just the tip of a disease iceberg. Below the surface of acute bacterial or viral infections lie chronic inflammations, the products of an immune system that is being constantly triggered by overexpressed cytokines. These triggers lead to the stimulation of different signaling pathways, which are instrumental in the development of chronic or “silent” inflammation. The signal messengers, such as the cytokines, carry instructions that are received by cells with specific receptors, which are able to detect them. Most dental procedures consist in eliminating acute inflammation in situations that do not feature typical signs of inflammation like pain and tissue swelling. This is the case with root fillings and surgical procedures like wisdom tooth surgery. The use of antibiotics helps the dentist and the patient overcome inflammation after dental procedures and during

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2 Evidence-Based Complementary and Alternative Medicine acute infections in daily practice. In daily dental practice, the effects of chronic inflammation on overall health are normally not of interest because local problems seem to be resolved after the symptoms of acute inflammation are gone. Consequently the individually targeted diagnosis of chronic pain in the peripheral facial nerves is a mostly neglected item in normal dental practice even though this sensory disturbance in particular has a strong negative impact on the quality of life of those who are affected. Peripheral nerves are the source of almost all forms of neuropathic pain. Neuropathic pain is a complex syndrome resulting from many different forms of peripheral nerve damage, such as traumatic nerve damage, diabetes, and infections, as well as immune system and metabolic diseases [6]. For decades, a neuron-centered argument has been frequently used to explain the pathophysiology of chronic pain; however, recent studies have shifted attention towards a neuroimmune

interaction. The concept of perineural jawbone inflammation producing or inducing facial neuralgia is an old one, and many oral surgical procedures have been recommended for “tic douloureux” [7]. This line of reasoning shifted when, in 1992, Bouquot examined 224 tissue samples from the mandibular alveolar bone of 135 patients with AFP or trigeminal neuralgia (TRN). All samples showed the clear presence of fatty-degenerative osteonecrosis spreading up to several centimeters in the form of retro-molar cavities in the cancellous bone. This brought Bouquot to propose the term “Neuralgia-Inducing Cavitation Osteonecrosis (NICO)” to describe the clinical phenomenon of neuralgia in conjunction with fatty-degenerative osteolysis and osteonecrosis of the jawbone (FDOJ) [8]. Further reports in the dental literature suggest that curettage of jawbone lesions is an effective treatment for the pain associated with a vascular FDOJ [9,10]. Notwithstanding these reports, the underlying effects of FDOJ on AFP/TRN remain unexplored by modern immunological means. In contrast to former destructive, intracranial, and extracranial ablative approaches to branches of the trigeminal nerve our hypothesis is that the reduction of acute inflammation might serve as the beginning of a possible development of chronic inflammation in jawbone. Persons with certain risk factors might be prone to developing subsequent chronic AFP/TRN. Although a multidisciplinary approach is required to address the many facets of this pain syndrome, no studies of AFP/TRN have established a connection between the direct role that cytokines and chemokines play in the pain-affected area or in pain syndromes of the jawbone. Elucidating the mechanisms, defining successful treatment strategies and a critical attitude to operation sites with insufficient wound healing in jawbone and treatments tailored to AFP/TRN is a crucial part of the here-presented therapeutic concept.

2. Materials and Methods 2.1. Patient Cohort. This study was performed as a randomized controlled trial. We collected FDOJ tissue samples from 15 patients with AFP/TRN. A diagnosis of AFP/TRN was made by neurologists, pain specialists, and physicians. Inclusion criteria were (1) therapy-resistant pain that was clinically similar to AFP/TRN and (2) the local diagnosis of FDOJ in the painful jaw site. Mandatory inclusion criteria were (3) the availability of two-dimensional orthopantomograms (2D-OPG) and (4) cone beam three-dimensional (digital volumetomograms DVT) images. A further inclusion criterion for the group with surgery in the AFP/TRN areas was the measurement of bone density of the jawbone with transalveolar ultrasound technology (TAU). Besides 2DOPG and 3D-DVT, the definite indication for FDOJ surgery was the additional measurement of bone density by TAU. TAU is a useful tool for establishing FDOJ [11–13]. Patients taking any medications due to neuropathic complaints were not excluded from the study. Demographic data from the AFP/TRN cohort showed an average age of 60 years (standard deviation (SD) = 13.2 years) and a gender ratio of 14:1 (female:male). The age range of the control group of 19 patients without FDOJ extended from 38 to 71 with an average age of 54 years and a gender split (female:male) of 11/8. This research was based on data retrieved from patients during normal dental surgery. All patients provided written informed consent. 2.2. Clinical Features of FDOJ Samples. The softening in FDOJ bone marrow is so distinct that the marrow space can be sucked and spooned out. Hollow cavitations with fatty degenerated adipocytes have undergone dystrophic changes

accompanied by demyelination of the bony sheath of the infra-alveolar nerve. All 15 FDOJ samples presented clinically and macroscopically as fatty lumps. FDOJ is similar to silent inflammation or subclinical inflammation without the typical signs of acute inflammation. Figure 1 shows a specimen with predominantly fatty transformation of the jawbone (a). The often-impressive extent of FDOJ lesions is illustrated in the right-hand panel by an X-ray with contrast medium. 2.3. Sampling of FDOJ Tissue. The current treatment of FDOJ lesions consists of curettage of the bony cavity, which relieves symptoms of pain with varying rates of success [8, 10, 14, 15]. To elucidate a possible causative link between FDOJ and AFP/TRN at the Munich Clinic for Integrative Dentistry, Germany, 15 patients with AFP/TRN and who were diagnosed with FDOJ had surgery on the affected area of the jaw. After local anesthesia and folding of a mucoperiosteal flap, the cortical layer was removed. All 15 patients exhibited FDOJ inside the bone marrow, which was quite similar to the samples described in the literature [8, 10]. In all 15 cases, surgery was performed on edentulous jaw areas in the sites of former wisdom teeth and the adjacent retro molar areas. The FDOJ samples, with a volume of up to 0.5 cm<sup>3</sup>, were stored in dry, sterile, 2 mL collecting vials (Sarstedt AG and Co, N<sup>u</sup>mbrecht, Germany), which were airtight, and frozen at -20°C. In addition to the cytokine analysis, we checked the FDOJ samples for pathohistological findings.

2.4. Processing of Necrotic Tissue Samples and Cytokine Measurements. In the examining Institute for Medical Diagnostics, Nicolaistraße 22, 12247 Berlin inspected by DAKKS (Deutsche Akkreditierungsstelle GmbH, accredited to DIN EN ISO/IEC 17025:2005 and DIN EN ISO 15189:2007), the samples were homogenized by mechanical force in 200 µL

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(a) (b)

Figure 1: FDOJ sample of fatty and osteolytic degenerated bone marrow (a) and contrast medium X-ray of the FDOJ cavity after curettage (b).

of cold protease inhibitor buffer (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics GmbH, Penzberg, Germany). The homogenate was then centrifuged for 15 minutes at 13,400 rpm. Next, the supernatant was collected and centrifuged for further 25 minutes at 13,400 rpm. In the 15 supernatants of tissue homogenate, we measured, regulated on activation, normal T-cells expressed and secreted (RANTES), also known as chemokine C-C motif ligand 5 (CCL5), FGF-2, interleukin- (IL-) 1 receptor antagonist (ra), IL-6, IL-8, monocyte chemoattractant protein-1 (MCP1), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Measurement was performed using the Human Cytokine/Chemokine Panel I (MPXHCYTO-60K; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions and analyzed using Luminex 200 with xPonent software (Luminex Co, Austin, TX, USA). 2.5. Pathohistological Examination. Parallel to the cytokine analysis, each FDOJ sample was examined histopathologically (Institute for Pathology & Cytology; Drs. Zwicknagel/Assmus, 85635 Freising, Germany).

3. Results As we showed in earlier publications [15, 16], the defining feature of the FDOJ areas is overexpression of the proinflammatory messenger RANTES, also known as CCL5. The results of the multiplex analysis of the seven cytokines in the AFP/TRN cohort ( $n = 15$ ) are shown in Table 1: AFP/TRN patients show elevated inflammatory signals in the FDOJ samples, deriving from painful jawbone areas with an average RANTES/CCL5 value of

4.274,7pg/mL (SD = 2.778pg/mL), compared to the randomized controlled sample of 149.9 (pg/mL) in healthy jawbone (Figure 2). All other cytokines were not derailed; only FGF-2 (fibroblast growth factor 2) and IL-1ra (interleukin 1 receptor antagonist) were additionally slightly upregulated in FJO samples.

Table 1: Pathohistological findings from FDOJ samples in 15 patients with AFP/TRN.

AFP/TRN 15 100% Ischemia 13 87% Necrotic adipocytes 10 67% Myxoid degeneration 12 80% Increased fat cells 12 80% Inflammatory cells 1 7%

In the pathohistological findings the amount of fat cells was consistently and strikingly increased in FDOJ samples. Typical signs of inflammation, especially of an inflammatory cell response, were absent. The fatty-degenerative and

osteolytic aspects occurred due to insufficient metabolic supply in

an ischemic state. The histologic examination of the curetted tissue demonstrated ischemia ( $n = 13$ ), necrotic adipocytes ( $n = 10$ ), myxoid degeneration ( $n = 12$ ), and increased fat cells ( $n = 12$ ); inflammatory cells were only found in one FDOJ sample. Table 1

shows the pathohistological findings in FDOJ samples from 15 patients with AFP/TRN.

4. Discussion 4.1. Histology in Neuropathic Facial Pain. The presence of inflammatory cells in only one FDOJ sample confirms inflammation-free progression and the absence of inflammatory granulation in FDOJ [17, 18]. This raises an important question: Are typical infections the underlying cause of chronic AFP/TRN? In summary, the pathohistological findings clearly show that AFP/TRN is not caused by an osteitic process that might produce typical symptoms like swelling and local inflammation; this is the likely reason why former attempts to diminish AFP/TRN by serial extraction of apical inflamed teeth exhibited poor success. In these cases,

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627.3

498.6

3.3 11.0 94.2 3.2

4.274,7

27.6

196.5 101.0 7.5 20.3 11 149.9 FGF-2 IL-1ra IL-6 IL-8 MCP-1 RANTES

AFP/TRN ( $n = 15$ ) Normal JB ( $n = 19$ )

TNF- $\alpha$

Figure 2: Analysis of seven cytokines in the FDOJ/AFP/TRN cohort ( $n = 15$ ) compared to healthy jawbones.

the alveolar jawbone remained untouched and the "silent inflammation" in the affected area continued unabated. FDOJ

must not be lumped together with other forms of osteomyelitis, which are defined by a dramatic increase in inflammatory cells.

4.2. Hyperactivated Chemokine RANTES/CCL5 in FDOJ. The absence of acute inflammation in FDOJ indicates that these chronic immunological processes are under the guidance of RANTES/CCL5, a proinflammatory chemokine. The hypothesis that FDOJ is an insidious, subtle process is supported by the fact that typical acute inflammatory cytokines, such as TNF- $\alpha$  and IL-6, were not increased in our samples. Proinflammatory cytokines have been repeatedly associated with demyelination and degeneration of the peripheral nerves, increased excitability of sensory afferents, and the induction

of neuropathic pain [19]. The significance of RANTES to the development of disease appears to be substantial: RANTES interferes with immune responses on a number of levels and therefore plays a crucial role in pathological states. The chemotactic properties of RANTES send T-cells, dendritic cells, eosinophils, natural killer (NK) cells, mast cells, and basophils to the sites of inflammation and infection [20]. RANTES is also an effective activator of leukocytes, which play a key role in a wide range of inflammatory disorders [21], including in rheumatoid arthritis [22] and diseases of the central nervous system, such as multiple sclerosis [23]. RANTES has also been associated with the induction or promotion of cancer [24]. RANTES levels were markedly elevated in the primary tumor and metastatic lesions of all patients with breast and cervical cancer in a previous study [25].

4.3. Origin of RANTES in FDOJ—Fatty Tissue and Adipocytes. Reduced blood flow and capillary density followed by ischemia may lead to a hypoxic environment [26]. Moreover, adipocytes and necrotic fat cells are considered immunologically effective ingredients. For instance, Huber et al. found increased expression of RANTES in fatty tissue in obese patients [27]. The role of these immune effects in understanding FDOJ, RANTES/CCL5, and facial pain is an evident issue that will be further illuminated later in the discussion. 4.4. Immunology in Neuropathic Facial Pain. Recent data suggest that there is a strong link between immune and glial cells and the development of neuropathic pain [19]. The present paper and other researches provide evidence that the nearly 30-fold overexpression of chemokine RANTES/CCL5

that we found in the painful jaw bone areas of the AFP/TRN cohort is linked to the disease development. Interactions between the immune and nervous systems occur at multiple levels, at which different types of immunologically active substances are involved in different stages of disease development [28]. Chronic pain is also associated with changes in neuroplasticity or changes in the neural pathways and synapses due to a defective reorganization of both the peripheral and central nervous systems. During tissue destruction, noxious stimuli and inflammation cause an increase in nociceptive input from the periphery to the central nervous system. Extended nociception from the periphery triggers a neuroplastic response at the cortical level and leads to a change in the somatotopic organization in the area of the body affected by pain; this results in central sensitization [19].

Moreover, immune activation near or around the peripheral nerves can cause increased excitability of these peripheral nerves. Both infectious substances and proinflammatory mediators may lead to changes in the blood-brain barrier (BBB) in response to chemotactic molecules that are released to the location of the damaged peripheral nerves which, in turn, leads neutrophils and macrophages to pass from the bloodstream into the nerves. Proinflammatory cytokines take part in this immune activation and shape the early immune response. However, these inflammatory mediators can directly increase nerve excitability, and they can cause damage to myelin and alter the permeability of the BBB. Furthermore, they can simultaneously lead to edema and further infiltration of the immune cells in peripheral nerves.

Schwann cells, which sheath the peripheral nerves, behave in a similar way to macrophages in the sense that they can present “non-self” substances to T-lymphocytes for further activation of immune cells. Schwann cells are also involved in the degradation of damaged myelin and cell debris [29]. Inflammatory mediators from the cells of the dorsal root ganglia (DRG), and those originating in the infiltrating

immune cells and activated spinal microglia, are key elements that carry signal transmission of the pain response [10]. 4.5. RANTES/CCL5 and Neuropathic Pain Syndromes. Cytokine/chemokine communication between glial cells and neurons is important for the development of neuropathic pain [30]. Studies indicate that prolonged chemokine and

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chemokine receptor activation in the sensory ganglia can significantly contribute to neuropathic pain syndromes.

Long-term chemokine inflow through RANTES/CCL5 causes neuronal hyper excitability. While proinflammatory cytokines, such as TNF- $\alpha$ , IL-6, and prostaglandins, are already distributed early in the acute stage of an injury or tissue infection, there are many indications that chemokines are activated at a later time, and they can act in the conversion of acute pain into a more chronic phenomenon. Recent data suggest that, in conjunction with tissue damage or infection, ischemia-induced chemokine expression causes an increase in inflammatory cytokines and thus leads to the hyper excitability of sensory neurons [31]. Since some chemokine receptors, such as CCR2, CCR5, CXCR4, and CX3CR1, are located mostly in the primary afferent neurons or secondary neurons of the dorsal spinal horn [32], their chemokine ligands may be able to alter the quality of pain transmission. By means of peripheral administration of the chemokines CCL2, CCL3, CCL5, and CXCL12, it is possible to detect pain patterns that are caused by the activation of chemokine receptors in dorsal root ganglia [33]. A study that examined the effects of CCR5 deficiency on pain responses by employing CCR5 knockout (KO) mice found that the pain responses of CCR5 KO mice to chemical or inflammatory stimuli were milder than those of CCR5 wild-type mice [34]. Another study examined the effects of CCR5 deficiency on pain responses via the use of CCR5 KO mice; it was observed that the pain responses of CCR5 KO mice to chemical or inflammatory stimuli were milder than those of the CCR5 wild-type mice [33]. 4.6. Opioid Receptors and Chemokine RANTES/CCL5. Recent studies have suggested that the chemokine RANTES and its receptor CCR5 interact directly with the opioid receptors and modify the nociceptive reaction [29]. Opioid receptors mediate antipain reactions, both in the peripheral and central nervous systems. The analgesic mechanism of morphine occurs when the analgesic opioid (e.g., morphine) excites the opioid receptors located in the brain and spinal cord; the perception of pain is blocked due to an agonistic, opposing effect. Morphine exerts its pain-relieving effect by binding to the nerve cells at the same binding sites as the endorphins; the specific binding sites are the opioid receptors. Fewer nociceptive neurotransmitters are released through morphine-induced opioid receptor excitation, and an incoming pain signal is not propagated. Studies have shown that opioid use suppresses chemokine-mediated chemotactic responses effectively, and this can be seen as a result of heterologous desensitization between opioids and some of the chemokine receptors [34]. The desensitization of opioid receptors through RANTES/CCL5 is part of this mutual "crossover" desensitization [35]. More recently, there have been reports showing that the process of heterologous desensitization is bidirectional, and that chemokine receptor activation leads to an inactivation of the in vitro activity of opioid receptors [36]. An open question that remains is whether some chemokines have the ability to desensitize opioid receptors in vivo. Studies using a rat model found that the analgesic response

was blocked in opioids following chemokine application [37, 38]. In these studies, Pizziketti et al. were able to show that proinflammatory chemokines, such as CCL2/MCP1, CCL5/RANTES, and CXCL8, are able to desensitize  $\mu$ -opioid receptors on the peripheral sensory neurons [39]. Therefore, these  $\mu$ -opioid receptors offer novel and potential mechanisms for peripheral inflammation-induced hyperalgesia. Scientists believe that this neural overexcitation materializes during chronic exposure to RANTES/CCL5 through the local overexpression in all trigeminal cases within the FDOJ and thus inhibits RANTES activity on the  $\mu$ -opioid receptors in the synapses. Moreover, chemokine-induced desensitization is mediated by the chemokine receptors [40].

Animals directly injected with specific doses of RANTES/CCL5 in the periaqueductal gray matter, a brain region that is the first to handle the antinociceptive effects of opioids, experience blocked and altered normal pain response to opioids. Our data indicate that proinflammatory chemokines are capable of desensitizing  $\mu$ -opioid receptors on peripheral sensory neurons, providing a novel potential mechanism for peripheral inflammation-induced hyperalgesia [40]. When the interval of the chemokine effect was extended to 2 hours, the ability of RANTES/CCL5 to desensitize opioid receptors was lost. A logical explanation for this is that the desensitization of opioid receptors is a reversible process that occurs via metabolic degradation. In our clinical neuralgia cases, the hypothesis of RANTES/CCL5 as a source of pain has persisted for years, so the experimental time limit for RANTES/CCL5 exposure on the opioid receptors is irrelevant. The above-cited experiments show that the opioid receptors can be desensitized by treatment with chemokines, which suggests that the desensitization of all three opioid receptors is achieved through the activation of RANTES/CCL5 [36]. Although RANTES/CCL5 desensitizes opioid receptors very effectively, desensitization does not work with all chemokines [41]. Recent studies have also shown that the chemokine/RANTES receptor CCR5 interacts with opioid receptors and leads to a change in the nociceptive reaction [42].

#### 4.7. Diagnostic Problems of FDOJ Lesions by X-Ray.

The nonvisible nature and lack of radiographic appearance of FDOJ make it difficult to obtain an accurate diagnosis [13]. Therefore, the existence of FDOJ is largely neglected today in mainstream dentistry. The reason for this is that conventional X-ray techniques are limited in their ability to reveal the actual extent and location of FDOJs. To aid the practitioner in diagnosing the debilitating effects of bone marrow softening inside FDOJ lesions, a computer-assisted TAU device was developed [43]. TAU precisely images and identifies cavitation porosity in the jawbone. Studies show that, in 84% of cases, FDOJ lesions on TAU images were more obvious and more readily identified than on radiographs of the same site. TAU imaging proved to be significantly superior to radiology for the detection of microscopically confirmed FDOJ. The efficiency and reliability of TAU in the diagnosis and imaging of FDOJ have been presented in earlier publications [44]. Because of these diagnostic difficulties, FDOJ as a presumably widespread jawbone disease is underdiagnosed by dentists in

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(a) (b) Figure 3: Curettage of FDOJ in the lower jaw with denuded infra-alveolar nerve. Corresponding X-ray without any signs of pathological process in jawbone (b).  
 general; specifically, in AFP/TRN cases, it may often falsely be referred to as “idiopathic.” The clinical example in Figure 3(a) shows the typical situation during surgical debridement and

curettage of the lower jaw. The infra-alveolar nerve is totally denuded from its bony sheath by FDOJ. The ischemic process of FDOJ converts the bony sheath, leaving the nerve tissue intact. As evidenced by what is not shown in the X-ray in the righthand panel of the figure, this process is inconspicuous and does not show any signs of inflammation or FDOJ. Because of this diagnostic problem of identifying FDOJ on common dental X-rays[13],thispatientsufferedfromAFPfor7years and received antidepressants during this time as a singular therapy.

4.8. Clinical Relevance of FDOJ Surgery in AFP/TRN Cases. The neurological theories and the data we retrieved from the FDOJ surgery resulted in pain relief in our AFP/TRN cohort. The subjective pain intensity in our AFP/TRN cohort was measured using the Numeric Rating Scale (NRS) [45]. The results of the NRS (ranging from 1 to 10) were changed into a percentage to evaluate pain relief. Figure 4 shows the mean time of AFP/TRN (45 months), the pain-free period after FDOJ curettage (21 months at the time the statistic was measured), and the overall percentage of pain relief (88%) in our 15 patients. Details of pain reduction in each patient are shown in Figure 5, which documents a mean percentage of 88%. Similar results in AFP/TRN pain relief were reported in other papers discussing FDOJ curettage [46].

4.9. A Clinical Case of FDOJ Surgery (Figure 6). To show the extent to which curettage of FDOJ in patients affected by AFP/TRN can contribute to alleviating facial pain and to give an example of the clinical relevance of FDOJ, our patient Mrs. N. T. reported the following: “Since spring 2009 I had been getting recurring stabbing pain on the left-hand side of my face and earache, tinnitus and pain in my shoulder/arm. During the night I suffered palpitations and panic attacks. My physical

45 months  
21 months  
88%

0 10 20 30 40 50 60 70 80 90 100 TRN/AFP before FDOJ surgery (months)  
Pain-free after FDOJ surgery (months)

Pain reduction in % after FDOJ surgery Figure 4: Mean time of AFP/TRN (45 months), the pain-free period after FDOJ curettage (21 months), and the overall percentage of pain relief (88%).

00

n=1 n=1

n=6

n=7

0-10 20-30 40-50 60-70 80-90 95-100 Percentage in pain reduction (n = 15 )

Figure 5: Percentage of pain reduction in the AFP/TRN cohort (n= 15).

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27.6 196.5 101.0 7.5 20.3 11 149.93 04.8 540.6 0.0 7.4 41.1 0.0

4.737,3

FGF-2 IL-1ra IL-6 IL-8 MCP-1 RANTES

Pat N.T. area 26 Norm (n = 19 )

TNF-α

Figure 6: A patient with AFP in the left upper jaw with overexpression of RANTES/CCL5 in the painful area. The corresponding X-ray

marked in red is inconspicuous; pain relief after FDOJ surgery was 90%.

energy levels also dropped. I consulted a further two dentists to no avail. One recommended that I went to see a neurologist, who prescribed me strong painkillers and psychotropic drugs. A trip to an osteopath was also unfortunately fruitless. In summer 2011 I was in a horrendous amount of pain, particularly at night. I could barely sleep through the night. I was taking strong painkillers every day just to get me to work. Then came the day when everything was solved. On 15 February 2012 I had an operation on the left side of my upper jaw and bone was excavated. After about 4 weeks I was almost pain-free without medication.”

5. Conclusions Although the role of proinflammatory cytokines and chemokines has been identified in neuropathic pain [38], the exact relationship between the chemokine–cytokine network and neuropathic pain is not fully understood. Jawbone cavitations are hollow dead spaces in the jaw bone, where the bone marrow is dying or dead. This research suggests that this jawbone disease, known popularly as “cavitations” and in some technical publications as “NICO,” might serve as a fundamental cause of neuropathic pain, through the inflammatory cytokines that it produces. Opioid receptors mediate anti-pain responses in both the peripheral and central nervous systems, and RANTES/CCL5 is able to enhance the pain response. As RANTES/CCL5 is overexpressed in jawbone areas defined by FDOJ, this process close to the trigeminal nerve might contribute to the development of AFP/TRN. Data from our research points to the local overexpression of RANTES/CCL5 in jawbones as a possible additional cause of AFP/TRN. Treatment for more advanced stages of FDOJ requires surgery. Surgical debridement of FDOJ areas can diminish RANTES/CCL5 overexpression and thus reduce chronic facial pain. The success of such surgery is by no means guaranteed and it depends on the technique and the skill of the dentist doing the surgery. FDOJ, as a contributing factor to AFP/TRN, is a widely neglected form of “silent inflammation” characterized by the overexpressed chemokine RANTES/CCL5. When doctors or dentists are presented with AFP/TRN of undetermined origin or that is “idiopathic,” a completed differential diagnosis should include FDOJ lesions. The presence of FDOJ is often not entirely obvious from examination of a panoramic or other X-rays. Many case histories in our clinic show that removing the diseased FDOJ from the jawbone may be the key to reversing the course of different forms of AFP/TRN. Further studies are needed to fully understand the neuropathic regulatory mechanisms that underlie neuroinflammation following nerve damage by cytokines deriving from FDOJ.

Disclosure Dr. VolkervonBaehr is the coauthor.

Conflict of Interests The authors declare that there is no conflict of interests regarding the publication of this paper.

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Aseptic-avascular osteonecrosis: local “silent inflammation” in the jawbone and RANTES/CCL5 overexpression

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**Abstract:** Of the definitions listed in the International Statistical Classification of Diseases and Related Health Problems, tenth revision (ICD-10), two disease descriptions can be found together: “idiopathic aseptic bone necrosis” and “avascular bone necrosis.” The relevant literature on both the conditions abbreviates both as “aseptic ischemic osteonecrosis in the jawbone” (AIOJ). To shed light on the clinical details of this condition, osteolytic jawbone samples of 24 patients with different systemic immunological diseases were examined using four steps: presurgical dental X-ray, postsurgical histology, polymerase chain reaction DNA analysis (PCR DNA) of bacteria, and RANTES/CCL5 (R/C) expression. These four steps showed that neither X-ray nor histology delivered unambiguous results with respect to inflammatory processes; furthermore, the PCR results did not show evidence of any microbial load within the jaw samples. However, there is a striking, coherent overexpression of chemokine R/C in the AIOJ samples. This study proved the aseptic existence of “silent inflammation” within the jawbone. The ICD-10 (AIOJ) definition, which is hard to interpret, can now be substantiated with clinical evidence, while the cytokine expressions described in this report can explain the systemic immunological effects observed within the group of examined patients. **Keywords:** jawbone, osteonecrosis, RANTES, CCL5, silent inflammation, aseptic inflammation, PCR, ICD-10

**Background** Chronic diseases with unknown triggers have been on the rise for years. Simultaneously, while medical science has been focusing on immunology, experts in the field are starting to realize that chronic inflammatory processes cause a vast number of diseases without the involvement of acute bacterial inflammation. Underlying these phenomena is an immune system that is being constantly triggered by overexpressed cytokines. These triggers lead to the stimulation of different signaling pathways, which are instrumental in the development of chronic disease.<sup>1</sup> At the same time, no other organ is exposed to such a large number of surgically invasive procedures than the teeth and jawbone area; for instance, tooth extractions during the mixed-dentition period, the surgical extraction of wisdom teeth, root canal filling on inflamed teeth, and dental implantations can all be accompanied by distorted wound healing. The persistent defects that result after healing, as well as insufficient bone regeneration as a form of “silent inflammation” in the dental area, are thus predestined.<sup>2</sup> These issues occur without the typical signs that usually accompany inflammation, and they thus often remain undetected. One must ask if it is possible that these cryptic processes

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can lead to immunological maladaptation, thereby causing erroneous signals and disease development.

**Study objectives** Code M87.0 of the International Statistical Classification of Diseases and Related Health Problems, tenth revision (ICD-10) presents the terms “idiopathic aseptic osteonecrosis” with the inclusion of “avascular osteonecrosis.”<sup>3</sup> Code M87.0 includes

cranial bones and the jawbone as well. The conceptual analysis of “idiopathic” depicts that a clear cause or infectious trigger is usually either not given or remains undetected. Within orthopedic practice and the relevant literature, those terms are often merged and referred to as “aseptic avascular osteonecrosis.” The term “avascular” is physiologically connected to a metabolic disorder accompanying ischemia and oxygen deficiency. The resulting decreased vascularization is a known possible cause of osteonecrosis. The guiding principles of the present analysis are to determine how the jawbone area is affected by bacterial ischemic and osteolytic processes. Furthermore, whether further chronic inflammatory phenomena are involved next to common acute inflammations in the jawbone such as chronic suppurative osteomyelitis, suppurative otitis, or bisphosphonate-induced osteonecrosis must be clarified. The present study aimed to elucidate these matters by using aseptic and thereby sterile clinical material and a model of inflammation without bacterial involvement.

**Materials and methods Patient collective** The present study was executed as a retrospective case control study and was deemed to be retrospective in nature and waived approval by IMD-Berlin (forensic accredited Institute DIN EN 15189/DIN EN 17025). All patients gave their written informed consent. This investigation is patient centered, as samples and data originated directly from daily practice. A total of 24 patients were included in this study. All patients were diagnosed with or treated for systemic immunological diseases or chronic pain in the trigeminus area to exclude the involvement of fatty degenerative osteonecrosis in the jawbone (FDOJ).<sup>4</sup> The patient population (n=24) was subdivided into four subgroups: atypical pain within the face and trigeminus area (n=4); neurodegenerative diseases (n=5, multiple sclerosis and amyotrophic lateral sclerosis); tumors (n=32, breast cancer and prostate cancer); rheumatism (n=11, fibromyalgia and Lyme disease); and chronic fatigue syndrome (CFS, n=5). Grounds for exclusion were acute osteonecrotic inflammatory reactions, the application of bisphosphonates due to their known impact on bone density, or a clinically proven diagnosis of osteoporosis. In addition to the presence of the aforementioned systemic diseases, participants were included in the study if they had a preoperative two-dimensional orthopantomogram (OPG), postoperative histology of FDOJ, or a polymerase chain reaction (PCR) pool sample of material taken intra-surgically from the FDOJ area, where the cytokine profile had additionally been examined with the help of multiplex analysis.

**Morphology and diagnostic criteria of FDOJ** In the FDOJ area, the bone material is irregular with thinned and hollowed out cancellous bone and there is also a medullary cavity. FDOJ is merely a medullary process; the cortex is usually undamaged and can clearly be distinguished from the hollowed and softened cancellous bone. The softening of the FDOJ medulla is so pronounced that the bone marrow space can indeed be sucked or spooned out. Therefore, from a morphological perspective, FDOJ are fatty lumps that can be extracted easily and without excessive bleeding from the bone marrow space. Trabecular structures of the cancellous bone have vanished almost entirely. The medullary bone lesion that arises and is microscopically most similar to FDOJ is ischemic aseptic osteonecrosis in the jawbone (IAOJ).<sup>5,6</sup> All the 24 FDOJ samples presented themselves clinically and macroscopically as fatty lumps. Figure 1A shows a sample of a medical preparation that features predominantly fatty alterations of the jawbone. Various types of FDOJ lesions are displayed using a contrast agent in Figure 2B. Several studies have shown that cases of

FDOJ are similar to those lesions that occur in the long bones and they stand out primarily due to the presence of ischemia, accompanying bone marrow edemas, and chronic non-suppurative osteomyelitis.<sup>7</sup> Light osteonecrosis does not tend to heal without surgical removal or curettage. FDOJ seems to mark the transition from an acute inflammation that arises following dental extraction to a chronically inflamed jawbone area that occurs instead of unimpeded wound healing. Sample collection and isolation of necrotic tissue samples In this study, 24 FDOJ patients underwent jaw surgery to one of the affected jaw areas. Following the administration of local anesthesia and the folding-down of one mucoperiosteal flap, the healthy cortical bone layer was removed to form a bone window. Within the bone marrow space, FDOJ was found in all patients. This form of osteonecrosis was similar

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to those samples described in the previous literature and is shown in Figure 1.8,9 In all cases, the procedure was executed in edentulous jawbone areas and adjacent retromolar fields. FDOJ samples with a volume of up to 0.5 cm<sup>3</sup> (1/2 cc) were extracted and immediately stored in a dry, sterile, airtight, 2 mL bottle (Sarstedt AG & Co, Nümbrecht, Germany) as a pea-sized tissue lump, which was then frozen at -20°C until being further transported.

Histology of the extracted bone samples Every FDOJ sample underwent histological examination at the Institute for Pathology & Cytology with Drs. Zwicknagel and Assmus (Freising, Germany). Figure 2 shows typical fatty marrow of FDOJ with mucoid degeneration and interstitial edema. Chronic degenerative changes are intermingled with foci of recent reactive adipocyte necrosis. The amount of fat cells is consistently and strikingly increased. Typical signs of inflammation, especially of an inflammatory cell response are missing. The fatty degenerative and osteolytic aspect overweighs due to insufficient metabolic supply. Marrow shows widened intertrabecular spaces that often contain small necrotic bone fragments, fatty microvesicles and pools of liquefied fat similar to oil cysts, with almost complete loss of adipocyte nuclei and residual fatty degenerated marrow and accumulation of acid mucopolysaccharides staining positively using alcianblue. Small nerve fibers are a striking feature in most biopsies of FDOJ, situated in close contact with degenerated and necrotic fatty tissue.

PCR DNA analysis of the extracted bone samples PCR is an artificial procedure that reproduces DNA. To detect possible bacterial colonization of the medullary osteolyses within the FDOJ area, we use a PCR procedure that is known from periodontology: obtaining the DNA proof of periodontopathogenic marker bacteria *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Campylobacter rectus/showae*, *Eikenella corrodens*, *Capnocytophaga gingivalis/ochracea*, *Parvimonas micra*, *Eubacterium nodatum*, and *Fusobacterium* spp. Since only one single FDOJ area is usually affected, we extract the characteristic medullary fatty lumps – as described in the “Sample collection and isolation of necrotic tissue samples” section – and insert four sterile paper sticks side by side (Figure

3). Those sticks remained in the area for 20 seconds and were then pooled and dispatched to the laboratory in a designated container.

Figure 1 (A) Morphology of fatty degenerative osteonecrosis in the jawbone. (B) Red circle indicates FDOJ area. Abbreviation: FDOJ, fatty degenerative osteonecrosis in the jawbone.

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A B

Figure 2 Pathohistological structure of typical fatty degenerative osteonecrosis in the jawbone tissue (200 fold).

HE, 200×

Figure 3 Paper sticks serving as a pooled sample within a fatty degenerative osteonecrosis in the jawbone lump. Abbreviation: FDOJ, fatty degenerative osteonecrosis in the jawbone.

Sterile paper pins

Sterile paper pins

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Analysis of presurgical radiodensity of the FDOJ area in OPG In normal dental practice, OPG is used to diagnose inflammation within the jawbone. To achieve an objective X-ray diagnosis of osteolytic FDOJ areas (X-ray typically being a visually performed method), we used OPG X-ray density (OPG-XrDn), which was executed using a KODAK 9000 Extraoral Imaging System X-ray unit in connection with the KODAK Dental Imaging Software 6.12.11.0; this method presented the results using a numerical quantity.<sup>10</sup> In the present study, only OPG-XrDn results achieved with OPG units from the author's practice are compared. At the jawbone areas that underwent surgery, an OPG-XrDn region of 1 cm was measured with a calibrated ruler. In Figure 4A, a normal retromolar jawbone area with an OPG-XrDn of 150 is shown, which corresponds with a normal jawbone in the XrDn.<sup>10</sup> Conversely, Figure 4B shows an extracted FDOJ sample, which is compared in size with a ceramic ball mill ( $\phi=0.6$  mm).

Reprocessing and cytokine/chemokine analysis of extracted FDOJ bone samples The parameter RANTES/CCL5 (R/C) in pg/mL was determined in the tissue homogenate supernatant on a Luminex® 200TM analysis unit with the help of xPonent® Software (Luminex, Austin, TX, USA) at the Institut für Medizinische Diagnostik Nicolaistr (Berlin, Germany).

Statistical methods The quantitative data obtained from the FDOJ group and the control group were analyzed using descriptive statistics, which were calculated using IBM SPSS, version 19 (IBM Corporation, Armonk, NY, USA). The median, the arithmetic mean value, and data distribution were calculated. Differences between cohorts were computed with Student's t-test or Spearman's rho. The two-sided unpaired t-test was used to determine the differences within groups, whereas Spearman's coefficient was used to analyze correlations among the examined cytokines. The significance level was set at  $P<0.05$ . Results Histology Based on the findings of those 24 FDOJ samples, a pathohistological definition for FDOJ can be developed that includes five distinct characteristics: "Undersupply in the form of a chronic trophic dysfunction"; "necrotically changed adipocytes"; "routinely significantly increased changed fat cells"; "myoxide

degeneration of fatty tissue”; and “inflammatory cells.” For the statistical analysis, we defined those five aforementioned terms. If an FDOJ sample contained all the five terms, it was rated as a “4”; if only three characteristics were evident, the sample received a score of “3”, and so forth. With respect to the histological results, none of these four characteristics would yield a score of “0”, which would indicate that a presurgical diagnosis of FDOJ is incorrect. In addition, the existence and intensity of inflammatory cells are taken into account. Histologically, three samples (12%) showed evidence of inflammatory processes, but in an inactive form, and they were thus not immediately inflammatory. There were no normal (healthy) samples, as each showed at least two of the characteristics described above. Fibrosis and ischemia – here described as “chronic trophic dysfunction” – were the most common results (Figure 5).

PCR analysis of FDOJ in terms of bacterial DNA Table 1 shows the results of the analysis of the 24 FDOJ samples. With the exception of samples 1 and 10, there was no evidence of bacterial colonization in any of the other samples. Table 2 shows the statistical results of periodontal pathogenic marker germs present in the samples.

Cytokine/chemokine analysis of FDOJ samples As we have shown in earlier studies, FDOJ areas are characterized by high levels of inflammatory messenger substance R/C, which is unlike normal jawbones.<sup>4</sup> A multiplex analysis of 19 comparable samples extracted from normal jawbones yielded R/C levels of 149 pg/mL (SD ±127) for these three cytokines. A search of the relevant literature did not yield corresponding results for these mediators in normal jawbone.<sup>4</sup>

Figure 4 (A) Measuring the OPG radiodensity of an FDOJ area in the lower jawbone, right-sided region =48/49. (B) Morphology of the extracted FDOJ area, which is compared in size with a ceramic ball mill ( $\phi=0.6$  mm). Abbreviations: òPG, orthopantomogram; XrDn, X-ray density; FDOJ, fatty degenerative osteonecrosis in the jawbone.

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Remarkably high divergences within the FDOJ collective stemmed from the hyper-activated signal transduction of R/C in 24 FDOJ samples. Figure 6 shows this inflammatory overexpression of the median R/C level (4,184.4 pg/mL, SD ±3,825.7); when comparing the four disease groups with the 19 normal jawbone samples (149.9 pg/mL of R/C),

Figure 5 Histological characteristics of the 24 FDOJ samples, of which only 12% showed “Inflammation-inactive cells.” Abbreviation: FDOJ, fatty degenerative osteonecrosis in the jawbone.

Inflammation-inactive 3

2.6

9

20

13

20

24

Grading

Myxoid degeneration

Fibrosis

Necrotic adipocytes

Trophic disturbance

Patients

Table 1 PCR DNA analysis of 24 FDOJ regions  
 Nr Re glo A a P g T f T d P i P m F s F n C r E n  
 E C C g 1 18/19 0 0 ++ ++ 0 0 +++ 0 +++ 0 0 0 2 48/49 0 + 0 0 0 0 0 0 0 0 ++ + 3 18/19 + 0 0 0  
 + 0 0 0 0 0 0 0 4 17 0 0 0 0 0 0 0 0 0 0 0 0 0 5 45/45 0 0 0 0 0 0 0 0 0 0 0 6 37 0 0 0 0 0 0 0  
 0 0 0 0 7 48/49 0 0 0 0 0 0 0 0 0 0 0 8 48/49 0 0 0 0 0 0 0 0 0 0 9 38/39 0 0 0 0 0 0 0 0  
 0 0 0 0 10 38/49 0 +++ +++ + 0 + ++ 0 + 0 0 0 11 48/49 0 0 0 0 0 0 0 0 0 0 0 12 16 0 0 0 0  
 0 0 0 0 0 0 0 13 28/29 0 0 0 0 0 0 0 0 0 0 0 14 48/49 0 0 0 0 0 0 + 0 0 0 0 15 18 0 0 0 0  
 0 0 0 0 0 0 0 16 36 0 0 0 0 0 0 + 0 0 0 0 17 38/39 0 0 0 0 0 0 0 0 0 0 0 18 28/29 0 0 0 0  
 0 0 0 0 0 0 0 19 24 0 0 0 0 0 0 0 0 0 0 20 18/19 0 0 0 0 0 0 0 0 0 0 21 38/39 0 0 0 0  
 0 0 0 0 0 0 0 22 48/49 0 0 0 0 0 0 0 0 0 0 23 48/49 0 0 0 0 0 0 0 0 0 0 24 48/49 0 0  
 0 0 0 0 0 0 0 0

Notes: Very high-degree pathogenic: *Aggregatibacter actinomycetemcomitans* (A a), *Porphyromonas gingivalis* (P g), *Tannerella forsythia* (T f), and *Treponema denticola* (T d). Highdegree pathogenic: *Prevotella intermedia* (P i), *Parvimonas micra* (P m), *Fusobacterium* spp. (F s), *Fusobacterium nucleatum* (F n), *Campylobacter rectus/showae* (C r), and *Eubacterium nodatum* (E n). Moderately pathogenic: *Eikenella corrodens* (E c) and *Capnocytophaga gingiva* (C g). + = moderate, ++ = strong, +++ = severe. Abbreviations: PCR DNA, polymerase chain reaction DNA analysis; FDOJ, fatty degenerative osteonecrosis in the jawbone.

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the following results were obtained: CFS, 6,078.4 pg/mL of R/C (n=6); neuralgiform complications, 6,130.3 pg/mL of R/C (n=6); breast cancer, 1,925.5 pg/mL of R/C (n=5); and rheumatoid complications, 2,591.4 pg/mL of R/C (n=7). XrDn analysis of FDOJ areas The mean value of the presurgical OPG-XrDn within the FDOJ areas of the analyzed collective (n=24) was 140.6 (SD ±28.83).

Correlations between radiodensity and RANTES levels in FDOJ samples The present data do not suggest that any significant correlations were present between radiodensity and RANTES levels; however, by excluding those three samples that were marked red in the diagram, the calculations resulted in an inverse correlation between radiodensity and R/C expression (r=-0.55; P=0.010). Remarkably, the three marked samples displayed the largest individual numeric differences between high measures of radiodensity (which would indicate a normal jawbone) and exuberant R/C signaling (Figure 7).

Summary of results Of the four parameters that we used for the diagnosis and clinically pathologic documentation of an intra-osseous inflammatory process, three (OPG, histology, and PCR DNA analysis) did not result in a positive reaction in the sense of a classic inflammatory reaction. Only ~30-times higher overexpression of R/C (pg/mL) in FDOJ samples was striking (Table 3).

Discussion This discussion centers on four clinical measurements that are used to evaluate the presence of IAOJ.6

Discussion of histological results The histological results indicate that minimal healing that was evident in FDOJ areas stemmed from local hypoxic, ischemic conditions in degenerative fatty structures. A critical balance between repairing and pro-inflammatory factors determines the clinical picture of incomplete wound healing in the jawbone following tooth or wisdom tooth surgeries, for instance. Incomplete wound healing is associated with four characteristic and reoccurring outcomes in FDOJ samples: 1) undersupply in the form of chronic trophic dysfunction. This serves as the basis for an ischemic metabolic state in FDOJ that – in consequence of a degenerative process – produces. 2) “Necrotically changed adipocytes” or “routinely significantly increased changed fat cells.” The intimate interaction between inflammatory cells and adipocytes is important, as it facilitates

Table 2 Statistical results of periodontal pathogenic marker germs present in the FDOJ samples A a P g T f T d P i P m F s F n C r E n E c C g 4% 16% 20% 12% 4% 4% 12% 0% 16% 0% 4% 4%

Notes: Very high-degree pathogenic: *Aggregatibacter actinomycetemcomitans* (A a), *Porphyromonas gingivalis* (P g), *Tannerella forsythia* (T f), and *Treponema denticola* (T d). Highdegree pathogenic: *Prevotella intermedia* (P i), *Parvimonas micra* (P m), *Fusobacterium* spp. (F s), *Fusobacterium nucleatum* (F n), *Campylobacter rectus/showae* (C r), and *Eubacterium nodatum* (E n). Moderately pathogenic: *Eikenella corrodens* (E c) and *Capnocytophaga gingiva* (C g). Abbreviation: FDOJ, fatty degenerative osteonecrosis in the jawbone.

Figure 6 Comparison of RANTES/CCL5 expression in 19 normal jawbone samples (blue column) and 24 FDOJ samples in four groups of ISD patients (red columns). Abbreviations: FDOJ, fatty degenerative osteonecrosis in the jawbone; ISD, immunological systemic disease.

Normal jawbone (n=19)

RANTES/CCL5 in FDOJ (pg/mL)

149.8

6,078.4

6,130.3

1,925.5

2,591.4

Chronic fatigue syndrome (n=6)

Neuralgic complaints (n=6)

Breast cancer (n=5)

Rheumatic complaints (n=7)

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Figure 7 Correlation between radiodensity and RANTES levels in FDOJ. The red lines indicate the mean values of FDOJ collective; the green lines are equivalent to values associated with a normal jawbone. Red dots indicate values out of statistical significant.

Abbreviations: XrDn, X-ray density; FDOJ, fatty degenerative osteonecrosis in the jawbone.

0

0

2,000  
 4,000  
 6,000  
 8,000  
 10,000  
 12,000 RANTES (pg/mL)  
 14,000  
 16,000  
 18,000  
 50 100 150 X-ray density  
 200 250

Mean RANTES (pg/mL) in FDOJ

Mean XrDn in FDOJ areas

Table 3 Overview and juxtaposition of the results obtained from 24 FDOJ samples from presurgical OPG radiodensity, intrasurgical R/C expression, histology, and PCR bacterial analysis of fatty degenerative necrotized medulla areas. Patient Area JB X-ray density RANTES (pg/mL) Histo inflammatcells (inactive) Histo ischam/fibros/ Troph/Necr Ad (grad) PCR bacteria (summary positive) 1 18/19 126 176 3 10 2 48/49 152 1172 3 1 3 18/19 156 1537 2 2 4 17 142 4035 3 0 5 45/46 90 4262 4 0 6 37 178 2575 Inactive 3 0 7 48/49 159 518 1 0 8 48/49 169 2087 3 0 9 38/39 122 677 Inactive 3 0 10 38/39 159 2000 1 8 11 48/49 146 2162 1 0 12 18/19 169 2917 3 0 f3 28/29 81 5352 2 0 14 48/49 147 17000 Inactive 2 0 15 18/19 132 4185 4 0 16 36 115 7036 3 0 17 38/39 122 10915 2 0 18 28/29 138 3122 3 0 19 24 101 3200 2 0 20 18/19 121 5600 2 0 21 38/39 146 2437 3 0 22 48/49 189 6950 2 0 23 48/49 192 9000 4 0 24 48/49 124 2125 3 0 Median 140 4210 2.6

Normal 125 149.9 Abbreviations: OPG, orthopantomogram; FDOJ, fatty degenerative osteonecrosis in the jawbone; R/C, RANTES/CCL5; PCR, polymerase chain reaction; JB, jawbone; Histo, histology; ischam, ischemia; fibros, fibrosis; troph, trophic dysfunction; Necr Ad, necrotic adipocytes; grad, grading.

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the secretion of inflammatory cytokines, which mediate the systemic effects associated with adipose tissue inflammation, which is described further in the “Discussion of PCR analysis of FDOJ’s bacterial DNA” section.<sup>11 3)</sup> As sign of malfunction within the FDOJ area’s micro-metabolism, a “characteristic myoxide degeneration of fat tissue” occurs, which is the histological equivalent of osseous structure disintegration in the FDOJ area. Finally, 4) only a few of the present inflammatory processes are inactive. Massively acutely produced inflammatory cells were not found in any of the 24 samples.

Radiodensity within the FDOJ area in OPG There is a need to discuss the extent to which the diagnostic instrument used most frequently by dentists – the two- dimensional panoramic X-ray – is suitable for the detection of aseptic ischemic osteonecrosis in the jawbone (AIOJ)/FDOJ. Any radiological tests are performed using the tester’s optic visual evaluation, which is a nonscientific, nonobjective procedure. The dangers associated with subjective misinterpretation are high. In radiological diagnostics, FDOJ areas seem to pose significant

problems for the dentist, which is why these conditions often remain unrecognized, in terms of both etiology and pathogenesis, as they take on the form of a “silent inflammation.”<sup>10</sup> Considerably, high limitations in the assessment of cancellous bone have to be taken into account.<sup>12,13</sup> Despite the recent progress that has been made in digital X-ray techniques and in improved medical imaging, problems regarding the display of cancellous bone remain.<sup>14</sup> Since FDOJ must not be confused with classic osteomyelitis and given that FDOJ has a largely unknown etiology, the literature has described problems with an “invisible” osteomyelitis in terms of its radiological appearance.<sup>15</sup> This form can be so subtle that a radiologist’s ability to detect it is close to impossible, unless the specialist has significant experience in diagnostics.<sup>16</sup> In the present study, the mean value of presurgical OPG-XrDn in the FDOJ areas of the analyzed sample (n=24) was 140.6 (SD ±28.83) with an average R/C expression of 4,184.4 pg/mL. In comparison, a normal jawbone expresses 120 KODAK units of RöDi and has an R/C expression of 149.9 pg/mL<sup>10</sup> (Figure 8). It can thus be concluded that optically evaluated OPG-XrDn does not indicate the presence of AIOJ/FDOJ.

Discussion of PCR analysis of bacterial DNA of FDOJ Since none of the analyzed samples demonstrated evidence of the bacterial colonization associated with periodontal pathogenic marker germs in the PCR pool samples (with the exception of two samples in which we suspected remnants were present in the extraction), then the notion that bacterial involvement as a microbial trigger occurs in FDOJ can be rejected. Apart from that, what else could serve as the inflammation carrier? The answer can be found in the “Discussion of the cytokine/chemokine analysis in FDOJ” section. We will discuss that signaling pathways are instrumental in the development of chronic disease.<sup>1</sup>

Discussion of the cytokine/chemokine analysis in FDOJ The messenger substances of immune system and healthy cancellous bone (n=19) Of the 19 participants included in this study, implant drills from normal jawbone were extracted. In a laboratory setting, those extractions were – as described in “Reprocessing and cytokine/chemokine analysis of extracted FDOJ bone samples” – tested for seven immunomediators. The mean values were as follows: fibroblast growth factor-2, 27.6 pg/mL; interleukin (IL)-1 ra, 196.5 pg/mL; IL-6, 101.0 pg/mL; IL-8, 7.5 pg/mL; MCP-1, 20.3 pg/mL; tumor necrosis factor (TNF)-a, 11.5 pg/mL; and R/C, 149.9 pg/mL.<sup>4</sup>

Hyperactivated R/C in osteolytic cancellous bone Since histology, PCR analysis, and OPG were unable to detect pathologic osseous processes, the most prominent contradiction that emerged stemmed from the sample population’s thirtyfold increase in the mean hyperactivated inflammatory

Figure 8 Comparison of RANTES/CCL5 expression and OPG X-ray density among the various samples collected between those with FDOJ and the normal results associated with a normal jawbone. Abbreviations: OPG, orthopantomogram; XrDn, X-ray density; FDOJ, fatty degenerative osteonecrosis in the jawbone.

4,184.4

149.9

RANTES/CCL5 X-ray density

140

FDOJ areas (n=24)

120

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R/C signaling within osteolytic areas. This constitutes the single positive parameter of a local inflammatory reaction. R/C is part of the chemotactic cytokine family (known as CC chemokines), which is reflected in the more modern description of CCL5. An early response gene synthesizes R/C; R/C is chemotactic for eosinophil granulocytes and basophilic cells and plays a key role in the recruiting process of leucocytes, which are at the center of inflammation. The importance of R/C with respect to disease occurrence seems to be enormous: R/C impairs the immunological reaction at many levels and therefore plays a key role in pathological pain. R/C's chemotactic characteristics send T-cells, dendritic cells, eosinophil granulocytes, natural killer cells, mast cells, and basophilic cells to the center of the inflammation.<sup>17</sup> In addition, R/C is a powerful leukocyte activator that is associated with many inflammatory complaints.<sup>18</sup> The tests performed on 24 FDOJ samples in this study unambiguously identified those areas as jawbones with a significant inflammatory burden, as these samples showed R/C values that were 30 times greater than those of normal jawbones (Figure 6).

Systemic impact of chemokine R/C Why is local R/C expression characteristic of AIOJ/FDOJ, and why is it of particular importance in the development of systemic immunological diseases? While acute inflammation during the wound-healing process constitutes an adaptive reaction via acute cytokines TNF- $\alpha$  and IL-6 with the aim of overcoming a disease, R/C overexpression takes place outside the normal reaction's framework and thus leads to a chronically maladaptive response.<sup>19</sup> Cytokine imbalances in the jawbone result in internal signal transmission due to the additional paths that stem from excess R/C. In the end, these paths may result in chronic pathologies such as cancer, diabetes, and cardiovascular diseases, as well as neurodegenerative and inflammatory processes – dependent on many other different implications. If a chronic disease is already present, the progressive deterioration that arises from undetected “silent inflammation” in the jawbone leads to a number of pathologic complications that have a negative impact on the overall condition of the jawbone and induce a vicious cycle of ongoing aggravation.<sup>20</sup> Furthermore, R/C is a powerful leukocyte activator that is of importance in another field of inflammatory malfunctions.<sup>17</sup> It affects several levels of the immune response and is thus substantially involved in infections or pathologic conditions. Most of the time, the defective regulation of R/C expression results in a selfreinforcing effect that can result in critical states in the human body, especially those that are in connection with diseases that affect the central nervous system, such as multiple sclerosis and Hodgkin's disease.<sup>21–23</sup> Immunohistological tests of various tissues showed that R/C is expressed only in small amounts in normal adult tissue. However, the proportion of R/C-positive cells increases dramatically, if inflammatory reactions occur.<sup>18</sup> In rheumatoid arthritis, the expression of R/C plays a vital role.<sup>24</sup> These findings underline that any of the immunologic systemic diseases that occurred in this sample might be linked to locally overexpressed R/C signaling.

The problem associated with radiological diagnoses of FDOJ lesions In the “Radiodensity within the FDOJ area in OPG” section, we demonstrated that FDOJ areas are invisible on X-

ray, rendering a precise traditional OPG-based diagnosis impossible. It follows that the existence of an FDOJ area and its meaning for the human organism are neglected in mainstream medicine and dentistry. The only limited possibilities associated with making a radiological diagnosis in connection with osseous edemas in FDOJ lesions necessitate bone density measurement by means of trans-alveolar ultrasound (TAU).<sup>25</sup> TAU very accurately and identifiably displays cavitation porosity in the jawbone and is thus more suitable for an FDOJ diagnosis than OPG. Due to these diagnostic inadequacies, FDOJ disorders often remain unrecognized by dentists.

**Summary** Here, we describe the overexpression of R/C in the jawbone with known diagnostic tools, including presurgical X-ray, intra-surgical histology, and PCR DNA analysis of bacterial colonization. Despite the negative test results obtained with OPG, the histological inflammatory parameters (no inflammatory cells traceable) and PCR parameters identified that the overexpression of R/C explains the possible systemic impact of “silent inflammation” in the IAOJ/FDOJ area. This led us to provide an overview of four inflammatory types in the jawbone, which were further subdivided based on X-ray visibility, bacterial involvement, pain, the associated R/C chemokine profile, and their systemic impact (Table 4).

**Conclusion** Using currently available methods, the present research shows that overactivated signal transduction cascades, especially of chemokine R/C, in osteolytic changes of the jawbone may be associated with the impacts of R/C on complex chronic diseases. The source of this inflammatory signaling is what we refer to as “fatty degenerative osteonecrosis in the jawbone” (FDOJ) due to its morphology, particularly given Clinical, Cosmetic and Investigational Dentistry 2017:9submit your manuscript | the presence of medullary cavities. Physiologically, FDOJ resembles asymptomatic AIOJ. By examining five primary factors, we can derive the following clinical conclusions:

1. Due to the absence of bacteria in an FDOJ/AIOJ area, a therapeutic prescription of antibiotics is futile.
2. OPG is not a sufficient method to exclude FDOJ/AIOJ during presurgical analysis.
3. For postsurgical analysis, a focus on histologically absent inflammatory cells is not sufficient; the classic “textbook” parameters of inflammation need to be expanded to include inflammatory mediators in the case of the jawbone, especially with respect to R/C.
4. The extension, which involves the chemokine expression of R/C in the intra-osseous definition of inflammation proposed here, may broaden a currently static perspective in dentistry and it may further add integrative dimensions.
5. To achieve the successful implementation of these perspectives, dentistry needs an analytic tool that is sufficient enough to display FDOJ/AIOJ and that is a complement to diagnostic radiology. Using clinical experience and research from the relevant literature, TAU is best for quantitatively determining bone density.<sup>26</sup>

As far as we are aware, this study is the first to clinically define “idiopathic avascular bone necrosis”, which is described in ICD-10 Code M87.0, as a carrier of so-called silent inflammation. Desirably, these subchronic inflammatory phenomena will receive more attention in mainstream medicine and dentistry, and it will be placed in context with systemic immunological diseases. Further studies are needed to fully understand the aseptic mechanisms in the jawbone that underlie chronic inflammation in systemic immune diseases following R/C overexpression in FDOJ.

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 development of MS, cancer and as a messenger in malignant cancer metastasis. Thus NICO  
 and RANTES give a possible complementary and integrative aspect of systemic diseases.  
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PO-048 Hospital-to-homes-mobility supported postoperative care management for surgical  
 wards

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Background: To reduce costs in the inpatient sector, hospitals are tending towards  
 reducing the duration of hospitalisation. With respect to demand- and resource-based  
 inpatient admissions and bed-occupancy planning, patient management systems have  
 proven their value and are established on surgical wards. Nevertheless, as the final link in  
 the process chain, patient-centred inpatient discharge procedures are not without  
 problems. In many cases, in the absence of provision for competent aftercare in the  
 patient's home, the situation becomes a crisis-management scenario, particularly for post-  
 operative surgery patients.

Methods: Solutions to this area of potential conflict have been developed by hospital-to-  
 homes mobile health services. These prototypical solutions were monitored and evaluated  
 during clinical application at a university hospital in cooperation with and with the  
 agreement of the regional association of doctors serving patients with statutory health  
 insurance, in this instance under the designation Medmobil. Results: The concept  
 developed by hospital-to-homes mobile health services enables inpatient discharge even  
 for complicated cases where potential risk exists (e.g. with wound-healing disorders). Data:  
 Patient recruitment n = 201/6month observation period, 87% visceral- and  
 transplantation surgery. Wound status and discharge points, 37% primary and 63%  
 secondary wound-healing disorders. Motivation to access Medmobil service; 96% return to  
 usual surroundings at home, 84% avoidance of unnecessary waiting time, 83% confidence  
 in the hospital service personal and 27% personal obligations. Assessment of quality of life  
 (SF-36). In summary, highly significant improvement in the general status (physically  
 4mentally, p<0.001). Conclusion of hospital-related treatment by Medmobil within the 14  
 days service period could be achieved in 52.7% of the patients. 47.2% required ongoing  
 medical supervision. Total amount of costs per visitation accounted 52h, in contrast to  
 daily hospital rate of 400h. There is no fragmentation in the provision of medical care and  
 the care requirements in the patient's home. The fragmentation of economically required  
 and surgically necessary just-in-time care and post-operative at-home care requirements  
 can be optimised in the interests of all parties at the end of the process chain. Under clinical  
 conditions, the concept solution from hospital-to-homes mobile health services is primarily  
 a patient-focused, logical

### 35. What statement is incorrect about saliva?

- Helps to form the enamel pellicle, on which healthy dental plaque can form.
- Provides active nitrates which eventually are metabolized to form nitric oxide to reduce blood pressure.
- Provides minerals to remineralize tooth surfaces.
- Assists in buffering to maintain a healthy pH of 3.5 in the dental plaque.

## Health benefits of saliva: a review.

- [Michael W J Dodds](#), [Dorthea A. Johnson](#), [Chih-Ko Yeh](#)
- Published in *Journal of dentistry* 2005
- Medicine

**OBJECTIVE** The aim is to present a review of the literature on human saliva composition, flow rates and some of the health benefits of saliva, with emphasis on studies from our laboratory that have looked at effects of age and age-related diseases on saliva output and composition. **DATA** Saliva influences oral health both through its non-specific physico-chemical properties, as well as through more specific effects. The proline-rich proteins, statherin and the histatins are salivary proteins that influence calcium phosphate chemistry, initial plaque formation and candida infection. Increases or decreases in mastication may affect saliva output. Our cross-sectional studies of saliva in a large population-based study cohort (N=1130) indicate that there is an age-related decline in saliva output for unstimulated whole, stimulated parotid, unstimulated submandibular/sublingual and stimulated submandibular/sublingual saliva, as well as some compositional alterations in anti-microbial and other proteins. Some of these alterations also appear to be specific for certain age-related medical conditions, such as diabetes mellitus. **CONCLUSIONS** These studies and data presented confirm the importance of saliva in maintaining a healthy oral environment; the practitioner is encouraged to consider saliva output and medical conditions that may compromise it as part of routine dental treatment planning. **LESS**

### From Nitrate to Nitric Oxide: The Role of Salivary Glands and Oral Bacteria

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**Abstract** The salivary glands and oral bacteria play an essential role in the conversion process from nitrate (NO<sub>3</sub><sup>-</sup>) and nitrite (NO<sub>2</sub><sup>-</sup>) to nitric oxide (NO) in the human body. NO is, at present, recognized as a multifarious messenger molecule with important vascular and metabolic functions. Besides the endogenous L-arginine pathway, which is catalyzed by complex NO synthases, nitrate in food contributes to the main extrinsic generation of NO through a series of sequential steps (NO<sub>3</sub><sup>-</sup>→NO<sub>2</sub><sup>-</sup>→NO pathway). Up to 25% of nitrate in circulation is actively taken up by the salivary glands, and as a result, its concentration in saliva can increase 10- to 20-fold. However, the mechanism has not been clearly illustrated until recently, when sialin was identified as an electrogenic 2NO<sub>3</sub><sup>-</sup>/H<sup>+</sup> transporter in the plasma membrane of salivary acinar cells. Subsequently, the oral bacterial species located at the posterior part of the tongue reduce nitrate to nitrite, as catalyzed by nitrate reductase enzymes. These bacteria use nitrate and nitrite as final electron acceptors in

their respiration and meanwhile help the host to convert nitrate to NO as the first step. This review describes the role of salivary glands and oral bacteria in the metabolism of nitrate and in the maintenance of NO homeostasis. The potential therapeutic applications of oral inorganic nitrate and nitrite are also discussed.

Keywords: nitrates, nitrate reductase, saliva, salivary physiology, nutrition/nutritional sciences, systemic health/disease

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reduction, platelet aggregation inhibition, and vasoprotective activity. In animal models, nitrate can protect against ischemiareperfusion injuries and several other types of cardiovascular disorders (Lundberg et al. 2011; Kapil et al. 2014).

**Nitrogen Species and the Cardiovascular System** The first description about the decrease of BP with dietary nitrate was reported in 2006 (Larsen et al. 2006). Moreover, interruption on the enterosalivary conversion of nitrate to nitrite can block the decrease of BP, confirming that nitrite converted from ingested nitrate can regulate BP (Webb et al. 2008).

In pulmonary hypertension models induced by hypoxia, nitrite acts as a pulmonary vasodilator, reduces right ventricular hypertrophy, and prevents pulmonary vascular remodeling (Baliga et al. 2012; Sparacino-Watkins et al. 2012). Cardiovascular benefits of nitrate presumably result from increasing vascular NO bioavailability (Kapil et al. 2010).

**Nitrate and Oxygen Utilization** Dietary inorganic nitrate relieves muscle fatigue and improves high-intensity exercise tolerance by reducing oxygen consumption and muscle metabolic perturbation during physical

exercise (Lansley et al. 2011; Khalifi et al. 2015). Mechanistically, dietary nitrate downregulates the expression of adenine nucleotide translocase and then improves skeletal muscle mitochondrial oxidative phosphorylation efficiency (i.e., phosphate/oxygen [P/O] ratio) and reduces oxygen consumption during exercise (Rolfe et al. 1994). In addition, NO derived from nitrate can interact with cytochrome C oxidase (complex IV) in competition with oxygen to partly slow down respiration (Larsen et al. 2011).

**Role of Sialin in Salivary Glands on Nitrate Transport from Blood to Saliva** The membrane protein sialin is a versatile anion transporter, and functional defects in the protein may have a deleterious impact on several critical physiologic functions (Reimer 2013). As a nitrate cotransporter in the salivary glands, sialin plays an important role in the physiologic regulation of systemic NO<sub>3</sub>--NO<sub>2</sub>--NO balance.

**Salivary Gland Physiology** Saliva is secreted by the 3 major salivary glands—including parotid, submandibular, and sublingual glands—with a slight contribution from many minor glands within the oral cavity (Melvin et al. 2005). Salivary flow is a continuous process that is upregulated by a reflex mostly stimulated by taste and chewing. The parotid gland is the largest gland and contributes the greatest flow (as much as 60% of the total) when stimulated by taste or chewing (Carpenter 2013). However, the submandibular and sublingual glands are less responsive to changes in diet and contribute more to the resting salivary flow rate (Reimer 2013).

**Sialin-Mediated Nitrate Transport** Sialin is a member of the SLC17 family, which is a group of 9 structurally related proteins that mediate the transmembrane transport of organic anions. The human SLC17 protein family includes the type I Na<sup>+</sup>/phosphate transporter

and 3 related proteins, 3 vesicular glutamate transporters, a vesicular nucleotide transporter, and sialin (Reimer and Edwards 2004). Sialin is expressed in all tissues (Aula et al. 2000), especially in parotid glands, brain, kidney, liver, pancreas, thyroid glands, and submandibular glands (Qin et al. 2012; Fig. 2). In salivary glands, sialin is located in the basolateral membrane of serous acinar cells and lysosomes (Qin et al. 2012; Fig. 2).

Figure 1. The role of salivary glands and oral bacteria in the  $\text{NO}_3^-$ - $\text{NO}_2^-$ - $\text{NO}$  pathway. Up to 25% of the circulating nitrate is actively taken up by the salivary glands and concentrated ~10- to 20-fold in the saliva to maintain the enterosalivary circulation of  $\text{NO}_3^-$ - $\text{NO}_2^-$ - $\text{NO}$ . This key process is mediated by sialin, which is an electrogenic  $\text{NO}_3^-/\text{H}^+$  transporter in the plasma membrane of salivary acinar cells. When saliva nitrate is secreted into the oral cavity with dietary nitrate—which is reduced to nitrite by the commensal facultative anaerobic bacteria at the posterior aspect of the tongue—some of the nitrite is converted into  $\text{NO}$  at the stomach. However, most of the remaining nitrate and nitrite are absorbed in the intestine and directly enter the systemic circulation, generating  $\text{NO}$  in blood and tissues under physiologic hypoxia and playing biological effects. *A. naeslundii*, *Actinomyces naeslundii*; *A. odontolyticus*, *Actinomyces odontolyticus*;  $\text{NO}$ , nitric oxide;  $\text{NO}_2^-$ , nitrite;  $\text{NO}_3^-$ , nitrate.

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From Nitrate to Nitric Oxide 3

As described above, there is a continuous uptake of nitrate from blood into the salivary glands (Lundberg et al. 2008). However, the exact mechanism of how nitrate is taken up by the salivary glands is largely unknown. Recently, the protein sialin has been identified as a nitrate transporter to mediate nitrate influx into the salivary gland by acting as an electrogenic  $2\text{NO}_3^-/\text{H}^+$  transporter in the plasma membrane of salivary acinar cells (Qin et al. 2012; Fig. 2). Knockdown of sialin expression reduces nitrate-transporting ability.

Fibroblasts from humans with mutations in the sialin gene have lower nitrate-transporting ability as compared with control groups. Sialin is endogenously localized in the lysosomes as well as in the plasma membrane of salivary gland cells. The plasma membrane sialin is a multifunctional anion transporter that can mediate electrogenic  $\text{A}^-/\text{H}^+$  cotransport of anions, such as sialic acid, glutamate, and aspartate. The function of sialin is assessed in vivo in pig salivary glands, and evidence for the physiologic relevance of sialin in mediating nitrate uptake  $\text{NO}_3^-$  influx into pig salivary glands was found (Qin et al. 2012). The nitrate transport discovery is important for future nitrate function studies, as the uptake of nitrate in the salivary glands and the subsequent excretion in saliva are necessary first steps for the conversion of nitrate to nitrite in the oral cavity (Lundberg 2012).

Role of Oral Bacteria on Nitrate Reduction to Nitrite Humans, unlike prokaryotes, are believed to lack the enzymatic machinery to reduce nitrate back to nitrite. However, due to the commensal bacteria that reside within the human body, it has been demonstrated that these bacteria can reduce nitrate,

thereby providing an alternative source of nitrite (Goaz and Biswell 1961; Takahashi 2015). Bacteria are vital in the process of converting nitrate to nitrite—the crucial first step in the  $\text{NO}_3^-$ - $\text{NO}_2^-$ - $\text{NO}$  pathway (Hezel and Weitzberg 2015).

Location of Nitrate-Reducing Bacteria in the Mouth After an oral nitrate loading, gastric  $\text{NO}$  concentration increases continually (McKnight et al. 1997). The importance of oral bacteria

in gastric NO generation has been clearly illustrated in experiments using germ-free sterile rats, in which gastric NO formation is negligible even after a dietary load of nitrate (Sobko et al. 2004). The experiment also showed that NO is very low in rats treated topically with an antiseptic mouthwash. Interestingly, the gastroprotective effects of dietary nitrate, discussed in the section below, virtually disappeared in rats treated with antiseptic mouthwash solutions (Petersson et al. 2009). The posterior surface of the tongue is responsible for the majority of nitrate reduction, while in the entire oral cavity the nitrate reduction is found to vary widely among individuals (Doel et al. 2005). Studies on rats have also shown that nitrate reductase activity occurs on the posterior surface of the tongue (Li et al. 1997) and that significantly higher proportions of gram-negative bacteria were found deep within the tongue clefts as compared with the surface.

**Composition of Nitrate-Reducing Bacteria** The major nitrate-reducing bacteria can be classified into 2 broad categories—the strict anaerobes (*Veillonella atypica* and *Veillonella dispar*) and the facultative anaerobes (*Actinomyces odontolyticus* and *Rothia mucilaginosa*; Doel et al. 2005; Table). The facultative anaerobe *A. odontolyticus* also displays markedly greater ability to reduce nitrate following incubation under anaerobic conditions. However, it is the strict anaerobes (*Veillonella* spp.) that have been found to be the most prevalent nitrate reducers on the tongue and therefore may be a major contributor to nitrate reduction in the oral cavity. Recently, by using 16S rRNA gene pyrosequencing and whole genome shotgun sequencing and analysis, scientists have found a higher abundance of *Prevotella*, *Neisseria*, and *Haemophilus* than *Actinomyces* on the posterior surface of the tongue (Hyde et al. 2014).

Figure 2. The expression and location of sialin. Sialin is expressed in parotid glands, brain, kidney, liver, pancreas, thyroid glands, and submandibular glands of miniature pig. Sialin is located in the serous acinar basolateral membrane of human salivary gland cells. mRNA, messenger RNA; NO<sub>3</sub><sup>-</sup>, nitrate.

Table. Composition of Nitrate Reductase Positive Isolates Identified from the Tongue.  
Bacteria %

*Veillonella atypica* 34 *Veillonella dispar* 24 *Actinomyces odontolyticus* 21 *Rothia mucilaginosa* 10 *Staphylococcus epidermidis* 5 *Rothia dentocariosa* 3 *Actinomyces naeslundii* 2

Data from Doel et al. 2005.

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**Role of Oral Mammalian Nitrate Reductase** The bacterial nitrate reductases include the following types: periplasmic, membrane bound, ferredoxin dependent assimilatory, and flavin dependent assimilatory (Sparacino-Watkins et al. 2014). Complex expression patterns are likely to be present among oral bacteria. In terms of nitrite production, respiratory denitrification—where nitrate is used as a terminal electron acceptor—is likely to be the major source of nitrite in the oral cavity (Li et al. 1997). These bacteria use nitrate as an alternative terminal electron acceptor during respiration to gain adenosine5-triphosphate in the absence of oxygen (Duncan et al. 1995; Li et al. 1997). The existence of a mammalian nitrate reductase is very promising because it could effectively reduce nitrate from food to nitrite. This nitrate-nitrite reduction plays an important role in the NO

generation from dietary nitrate. The nitrate reductase will be a suitable target for new drug research, yet its role in normal regulation of nitrite and NO homeostasis remains to be illustrated. A marked attenuation of the nitrate-induced plasma nitrite peak was noted in subjects that rinsed their mouth with an antibacterial mouthwash immediately prior to nitrate ingestion (Govoni et al. 2008). It seems that most nitrite generates rapidly in humans after a nitrate load is derived from the bacterial pathway described above.

**Saliva Nitrate Protecting against Gastric Damage** Nitrate secreted from the salivary glands is found to have an unprecedented function in protecting against stress-induced gastric damage (Jansson et al. 2007). A water immersion– restraint stress assay in rats shows decreased blood flow in gastric mucosa and induced hemorrhagic erosions after bilateral parotid and submandibular duct ligation (Jin et al. 2013). In animals that had received either cardiac ligation or oral treatment with povidone-iodine, a potent bactericidal agent, administration of nitrate failed to increase gastric levels of NO and to inhibit the mucosal injury (Miyoshi et al. 2003). NO that is formed close to the gastric mucosa can easily diffuse through the mucosa to the submucosal arterioles, causing vasodilatation and thus increasing gastric mucosal blood flow (Björne et al. 2004). This process protects gastric epithelial cells from necrosis. In addition, the decrease of mucosal myeloperoxidase activity and the expression of induced NO synthase with nitrate pretreatment imply that nitrate can reduce tissue inflammation, making this mechanism a possible way of gastric protection (Björne et al. 2006). In the absence of a dietary nitrate intake, salivary nitrate originates mainly from NO synthase. Thus, oxidized NO from the endothelium and elsewhere is recycled to regulate gastric mucus homeostasis (Petersson et al. 2015).

**Oral Bacterial Nitrate in Oral Diseases** Total salivary nitrates and nitrites are higher in patients with periodontal disease. The increase in nitrate secretion and a subsequent increase in salivary nitrite may contribute to the overall protective effect against those infections conditions. It is known that salivary glands may respond to periodontitis by enhancing the protective potential of saliva (Henskens et al. 1996). It is likely that the increment of salivary nitrate/nitrite concentration in patients with periodontal disease is due to an increase in nitrate secretion as a response of salivary glands to the inflammatory process. Because nitrate is described as the basis of a non-immune system mediated defense mechanism against gastrointestinal and oral pathogens in animals and humans (Duncan et al. 1997), its increase in patients with periodontal disease is considered to be associated with the host defense reaction (Sanchez et al. 2014). Indeed, in a previous study, a significant reduction in caries experience was found in patients with high salivary nitrate concentration and high nitrate-reducing ability when compared with the control subjects (Doel et al. 2004).

**Future Research into the Functions of the Oral Bacterial and Salivary Gland on Nitrate Reduction** In this review, we present the enterosalivary circulation of NO<sub>3</sub>-NO<sub>2</sub>--NO and the physical function of inorganic nitrate and nitrite. The salivary glands and oral bacteria play an important role in maintaining NO homeostasis in the human body. Dysfunction of salivary glands and oral bacteria may lead to a decrease of NO generation from the enterosalivary pathway of NO<sub>3</sub>--NO<sub>2</sub>--NO. However, whether nitrate and/or nitrite can play a physiologic role independent of NO still needs to be investigated. As for salivary gland-mediated nitrate transport, studies need to be carried out to illustrate how nitrate is secreted from acinar cells into saliva and to analyze the protein structure of sialin. It will be of interest to study the exact mechanism of nitrate transport by sialin in different kinds of

cells and to investigate if sialin transports other anions, such as perchlorate, iodine, and glutamate, from blood into saliva. The relationship between nitrate reductase and human diseases should be determined to obtain basic data for the clinical application of nitrate. Author Contributions X.M. Qu and Z.F. Wu, contributed to data acquisition, and interpretation, drafted and critically revised the manuscript; B.X. Pang and L.Y. Jin contributed to data acquisition, interpretation, and drafted the manuscript; L.Z. Qin contributed to conception and design, critically revised the manuscript; S.L. Wang contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

### 36. Which statement is incorrect?

- Local antimicrobials used daily can prevent mouth ulcers and promote balance in the oral microbiome.
- The human gut contains about 38 trillion bacteria cells, and the human body contains about 30 trillion human cells.
- Gut dysbiosis can cause lesions in the mouth.
- Diet and lifestyle changes could prevent and heal mouth lesions.

#### Revised Estimates for the Number of Human and Bacteria Cells in the Body

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#### Abstract

Reported values in the literature on the number of cells in the body differ by orders of magnitude and are very seldom supported by any measurements or calculations. Here, we integrate the most up-to-date information on the number of human and bacterial cells in the

body. We estimate the total number of bacteria in the 70 kg "reference man" to be  $3.8 \times 10^{13}$ .

For human cells, we identify the dominant role of the hematopoietic lineage to the total count ( $\approx 90\%$ ) and revise past estimates to  $3.0 \times 10^{13}$  human cells. Our analysis also updates the widely-cited 10:1 ratio, showing that the number of bacteria in the body is actually of the same order as the number of human cells, and their total mass is about 0.2 kg.

**Introduction** How many cells are there in the human body? Beyond order of magnitude statements that give no primary reference or uncertainty estimates, very few detailed estimates have been performed (the one exception [1] is discussed below). Similarly, the ubiquitous statements regarding  $10^{14}$ – $10^{15}$  bacteria residing in our body trace back to an old back-of-the-envelope calculation [2–4]. The aim of this study

istocritically revisit former estimates for the number of human and bacterial cells in the human body. We give up-to-date detailed estimates where the calculation logic and sources are fully documented and uncertainty ranges are derived. By updating the cell counts in the body, we also revisit the 10:1 value that has been so thoroughly repeated as to achieve the status of an established common knowledge fact [4]. This ratio was criticized recently in a letter to the journal *Microbe* [5], but an alternative detailed estimate that will give concrete values and estimate the uncertainty range is needed. Here, we provide an account of the methodologies employed hitherto for cell count and revise past estimates. Doing so, we repeat and reflect on the assumptions in previous back-of-the-envelope calculations, also known as Fermi problems. We find such estimates as effective sanity checks and a way to improve our quantitative understanding in biology. A major part of the available literature used in the derivation of human cell numbers was based on cohorts of exclusively or mostly men, and as we use these sources, our analysis starts

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Abbreviations: CV, coefficient of variation; FISH, fluorescent in situ hybridization; SD, standard deviation; SA, surface area; SEM, Standard Error of the Mean.

with adult men. As discussed below, relatively modest quantitative differences apply for women due to changes in characteristic body mass, blood volume, and the genital microbiota. For our analysis, we used the definition of the standard reference man as given in the literature [6] as: "Reference Man is defined as being between 20–30 years of age, weighing 70 kg, is 170 cm in height." Our analysis revisits the estimates for the number of microbial cells, human cells, and their ratio in the body of such a standard man. We begin our analysis by revisiting the number of bacteria through surveying earlier sources, comparing counts in different body organs and finally focusing on the content of the colon. We then estimate the total number of human cells in the body, comparing calculations using a "representative" cell size to aggregation by cell type. We then contrast the cell number

distribution by tissue type to the mass distribution. In closing, we revisit the ratio of bacterial to human cells and evaluate the effect of gender, age, and obesity.

**Results** Origin of Prevalent Claims in the Literature on the Number of Bacterial Cells in Humans  
Microbes are found throughout the human body, mainly on the external and internal surfaces, including the gastrointestinal tract, skin, saliva, oral mucosa, and conjunctiva. Bacteria overwhelmingly outnumber eukaryotes and archaea in the human microbiome by 2–3 orders of magnitude [7,8]. We therefore sometimes operationally refer to the microbial cells in the human body as bacteria. The diversity in locations where microbes reside in the body makes estimating their overall number daunting. Yet, once their quantitative distribution shows the dominance of the colon as discussed below, the problem becomes much simpler. The vast majority of the bacteria reside in the colon, with previous estimates of about  $10^{14}$  bacteria [2], followed by the skin, which is estimated to harbor  $\sim 10^{12}$  bacteria [9]. As we showed recently [4], all papers regarding the number of bacteria in the human gastrointestinal tract that gave reference to the value stated could be traced to a single back-of-the-envelope estimate [3]. That order of magnitude estimate was made by assuming  $10^{11}$  bacteria per gram of gut content and multiplying it by 1 liter (or about 1 kg) of alimentary tract capacity. To get a revised estimate for the overall number of bacteria in the human body, we first discuss the quantitative distribution of bacteria in the human body. After showing the dominance of gut bacteria, we revisit estimates of the total number of bacteria in the human body.

**Distribution of Bacteria in Different Human Organs** Table 1 shows typical order of magnitude estimates for the number of bacteria that reside in different organs in the human body. The estimates are based on multiplying measured concentrations of bacteria by the volume of each organ [9,10]. Values are rounded up to give an order of magnitude upper bound. Although the bacterial concentrations in the saliva and dental plaque are high, because of their small volume the overall numbers of bacteria in the mouth are less than 1% of the colon bacteria number. The concentration of bacteria in the stomach and the upper 2/3 of the small intestine (duodenum and jejunum) is only  $10^3$ – $10^4$  bacteria/mL, owing to the relatively low pH of the stomach and the fast flow of the content through the stomach and the small intestine [10]. Table 1 reveals that the bacterial content of the colon exceeds all other organs by at least two orders of magnitude. Importantly, within the alimentary tract, the colon is the only substantial contributor to the total bacterial population, while the stomach and small intestine make negligible contributions.

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Revisiting the Original Back-of-the-Envelope Estimate for the Number of Bacteria in the Colon

The primary source for the often cited value of  $\sim 10^{14}$  bacteria in the body dates back to the 1970s [3] and only consists of a sentence-long “derivation,” which assumes the volume of the alimentary tract to be 1 liter, and multiplies this volume by the number density of bacteria, known to be about  $10^{11}$  bacteria per gram of wet content. Such estimates are often very illuminating, yet it is useful to revisit them as more empirical data accumulate. This pioneering estimate of  $10^{14}$  bacteria in the intestine is based on assuming a constant bacterial density over the 1 liter of alimentary tract volume (converting from volume to mass via a density of 1 g/mL). Yet, the parts of the alimentary tract proximal to the colon contain a negligible number of bacteria in comparison to the colon content, as can be appreciated from Table 1. We thus conclude that the relevant volume for the high bacterial density of  $10^{11}$

bacteria/g is only that of the colon. As discussed in Box 1, we integrated data sources on the volume of the colon to arrive at 0.4 L.

The Total Number of Bacteria in the Body We are now able to repeat the original calculation for the number of bacteria in the colon [3]. Given  $0.9 \times 10^{11}$  bacteria/g wet stool as derived in Box 2 and 0.4 L of colon, we find  $3.8 \times 10^{13}$  bacteria in the colon with a standard error uncertainty of 25% and a variation of 52% SD over a population of 70 kg males. Considering that the contribution to the total number of bacteria from other organs is at most  $10^{12}$ , we use  $3.8 \times 10^{13}$  as our estimate for the number of bacteria across the whole body of the "reference man." We note that the uncertainty estimate value takes into account known variation in the colon volume, bacteria density, etc., but cannot account for unquantified systematic biases. One prominent such bias is the knowledge gap on differences between the actual bacteria density in the colon, with all its spatial heterogeneity, and the measurements of concentration in feces, which serve as the proxy for estimating bacteria number. What is the total mass of bacteria in the body? From the total colon content of about 0.4 kg and a bacteria mass fraction of about one-half [21,24], we get a contribution of about 0.2 kg Table 1.

Bounds for bacterial number in different organs, derived from bacterial concentrations and volume. e. Location Typical concentration of bacteria (1) (number/mL content) Volume (mL)

Order of magnitude bound for bacterial number Colon (large intestine)  $10^{11}$  400 (2)  $10^{14}$   
Dental plaque  $10^{11}$   $<10^{10}$   $10^{12}$  Ileum (lower small intestine)  $10^8$  400 (5)  $10^{11}$  Saliva  $10^9$   
 $<10^6$   $10^{11}$  Skin  $<10^{11}$   $\text{cm}^2$  (3)  $1.8\text{m}^2$  (4)  $10^{11}$  Stomach  $10^3$ – $10^4$  250 (5)–900 (6)  $10^7$   
Duodenum and Jejunum (upper small intestine)  $10^3$ – $10^4$  400 (5)  $10^7$

(1) Except for skin, concentrations are according to [9]. For the skin, we used bacterial area density and total skin surface to reach an upper bound. (2) Seed derivation in section below. (3) Skin surface bacterial density is taken from [11]. (4) Skin area calculated as inferred from standard formula by DuBois for the body surface area [12]. (5)

Volume of the organs of the gastrointestinal tract is derived from weight taken from [13] by assuming content density of 1.04 g/mL [6]. (6) High value is given in [14].

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(wet weight) from bacteria to the overall mass of the colon content. Given the dominance of bacteria in the colon over all other microbial populations in the body, we conclude that there is about 0.2 kg of bacteria in the body overall. Given the water content of bacteria, the total dry weight of bacteria in the body is about 50–100 g. This value is consistent with a parallel alternative estimate for the total mass of bacteria that multiplies the average mass of a gut bacterium of about 5 pg (wet weight, corresponding to a dry weight of 1–2 pg, see S1 Appendix) with the

updated total number of bacteria. We note that this empirically observed average gut bacterium is several times bigger than the conveniently chosen "standard"  $1 \mu\text{m}^3$  volume and 1 pg wet mass bacterium often referred to in textbooks. The total bacteria mass we find represents about 0.3% of the overall body weight, significantly updating previous statements that 1%–3% of the body mass is composed of bacteria or that a normal human hosts 1–3 kg of bacteria [25].

Box 1. The Volume of the Human Colon Content This is a critical parameter in our calculation. We used a value of 0.4 L based on the following studies (see also S1 Data, tab Colon Content). The volume of the colon content of the reference adult man was previously estimated as 340 mL (355 g at density of 1.04 g/mL [6]), based on

various indirect methods including flow measurements, barium meal X-ray measurements and postmortem examination [13]. A recent study [15] gives more detailed data about the volume of undisturbed colon that was gathered by MRI scans. The authors report a height-standardized colonic inner volume for males of  $97 \pm 24 \text{ mL/m}^3$  (where the best fit was found when dividing the colonic volume by the cube of the height). Taking a height of 1.70 m for the reference man [6], we arrive at a colon volume of  $480 \pm 120 \text{ mL}$  (where unless noted otherwise  $\pm$  refers to the standard deviation [SD]). This volume includes an unreported volume of gas and did not include the rectum. Most recently, studies analyzing MRI images of the colon provided the most detailed and complete data. The inner colon volume in that cohort was 760 mL in total [16,17]. This cohort was, however, significantly taller than the reference man. Normalizing for height, we arrive at 600 mL total volume for a standard man. In order to deduct the volume occupied by gas, stool fraction in this report was estimated at  $\approx 70\%$  of colon volume leading to 430 mL of standardized wet colon content. Therefore, this most reliable analysis together with earlier studies support an average value of about 0.4 L. We can sanity-check this volume estimate by looking at the volume of stool that flows through the colon. An adult human is reported to produce on average 100–200 grams of wet stool per day [18]. The colonic transit time is negatively correlated with the daily fecal output, and its normal values are about 25–40 hours [18,19]. By multiplying the daily output and the colon transit time, we thus get a volume estimate of 150–250 mL, which is somewhat lower than but consistent with the values above, given the uncertainties and very crude estimate that did not account for water in the colon that is absorbed before defecation. To summarize, the volume of colon content as evaluated by recent analyses of MRI images is in keeping with previous estimates and fecal transit dynamics. Values for a reference adult man averaged 0.4 L (standard error of the mean [SEM] 17%, coefficient of variation [CV] 25%), which will be used in calculations below. Following a typical meal, the volume changes by about 10% [15], while each defecation event reduces the content by a quarter to a third [18].

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**The Number of Human Cells in a “Standard” Adult Male** Many literature sources make general statements on the number of cells in the human body ranging between  $10^{12}$  to  $10^{14}$  cells [26,27]. An order of magnitude back-of-the-envelope argument behind such values is shown in Box 3.

**Box 2. Concentration of Bacteria in the Colon** The most widely used approach for measuring the bacterial cell density in the colon is by examining bacteria content in stool samples. This assumes that stool samples give an adequate representation of colon content. We return to this assumption in the discussion. The first such experiments date back to the 1960s and 1970s [20,21]. In those early studies, counting was based on direct microscopic clump counts from diluted stool samples.

Later experiments [22,23] used DAPI nucleic acid staining and fluorescent in situ hybridization [FISH] to bacterial 16S RNA. Values are usually reported as bacteria per gram of dry stool. For our calculation, we are interested in the bacteria content for the wet rather than dry content of the colon. To move from bacteria/g dry stool to bacteria/g wet stool we use the fraction of dry matter as reported in each article. Table 2 reports the values we extracted from 14 studies in the literature and translated them to a common basis enabling comparison. Table 2. Values of bacterial density in stool as reported in several past articles. Article bac.#/g dry stool ( $\times 10^{11}$ ) dry matter as % of stool bac.#/g wet stool ( $\times 10^{11}$ ) CV(%) Author Year

Houte&Gibbons 1966 - - 3.2 53% Moore&Holdeman 1974 5 22% 1.1 78%  
 Holdeman,Good& Moore 1976 4.1 31% 1.3 66% Stephen&Cumplings 1980 4 29%(1) 1.2  
 25% Langendijk etal. 1995 - - 2.7 26% Franksetal. 1998 2.9 - 0.74(2) 39%  
 Simmering&Kleessen 1999 4.8 - 1.3(2) 44% Tannocketal. 2000 - - 0.95 40%  
 Harmsen,Raangs,He,Degener& Welling 2002 2.1 30% 0.62 38% Zoetendaletal 2002 2.9 -  
 0.77(2) 24% Zhongetal. 2004 1.5 23% 0.35 73% Thiel& Blaut 2005 3.5 25% 0.87 53%  
 Heetal. 2008 1.5 - 0.39(2) 43% Uyeno,Sekiguchi&Kamagata 2008 - - 0.44 34% Mean - 27%  
 $\pm 2\%$   $0.92 \pm 19\%$  46%

Full references are provided in Table A in S1 Appendix. Mean bacterial number is calculated using the geometric mean to give robustness towards outlier values. Values quoted directly from the articles are written in bold, values derived by us are written in italic. Values reported with more than two significant digits are rounded to two significant digits as the uncertainty makes such over specification nonsense.  $\pm$  standard error of the mean. (1) Value for [21] derived from their Table 1. (2) From derivation, assuming the averaged dry matter fraction of 27%.

From the measurements collected in Table 2, we calculated the representative bacteria concentration in the colon by two methods, yielding very close values: the geometric mean is  $0.92 \times 10^{11}$  (SEM 19%) bacteria per gram of wet stool, while the median of the values is  $0.91 \times 10^{11}$  (SEM 19% by bootstrapping, see methods in S1 Appendix). The variation across the population, given by the average CV, is 46%.

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An alternative method that does not require considering a representative "average" cell systematically counts cells by type. Such an approach was taken in a recent detailed analysis [1]. The number of human cells in the body of each different category (by either cell type or organ system) was estimated. For each category, the cell count was obtained from a literature reference or by a calculation based on direct counts in histological cross sections. Summing over a total of 56 cell categories [1] resulted in an overall estimate of  $3.7 \times 10^{13}$  human cells in the body (SD  $0.8 \times 10^{13}$ , i.e., CV of 22%). Updated Inventory of Human Cells in the Body In our effort to revisit the measurements cited, we employed an approach that tries to combine the detailed, census approach with the benefits of a heuristic calculation used as a sanity check. We focused on the six cell types that were recently identified [1] to comprise 97% of the human cell count: red blood cells (accounting for 70%), glial cells (8%), endothelial cells (7%), dermal fibroblasts (5%), platelets (4%), and bone marrow cells (2%). The other 50 cell types account for the remaining 3%. In four cases (red blood cells, glial cells, endothelial cells, and dermal fibroblasts), we arrived at revised calculations as detailed in Box 4. Our revised calculations of the number of glial cells, endothelial cells, and dermal fibroblast yield only  $0.9 \times 10^{12}$  cells, in contrast to  $7.5 \times 10^{12}$  cells in the previous estimate. This leaves us Box 3. Order of Magnitude, Naïve Estimate for the Number of Human Cells in the Body Assume a 102 kg man, consisting of "representative" mammalian cells. Each mammalian cell, using a cell volume of  $1,000\text{--}10,000 \mu\text{m}^3$ , and a cell density similar to that of water, will weigh  $10\text{--}12\text{--}10\text{--}11 \text{ kg}$ . We thus arrive at  $10^{13}\text{--}10^{14}$  human cells in total in the body, as shown in Fig 1. For these kind of estimates, where cell mass is estimated to within an order of magnitude, factors contributing to less than 2-fold difference are neglected. Thus,

we use 100 kg as the mass of a reference man instead of 70 kg and similarly ignore the contribution of extracellular mass to the total mass. These simplifications are useful in making the estimate concise and transparent.

Fig 1. Back of the envelope estimate of the number of cells in an adult human body based on a characteristic volume and mass.

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with  $3.0 \times 10^{13}$  human cells in the 70 kg “reference man,” with a calculated 2% uncertainty and 14% CV. We note that the uncertainty and CV estimates might be too optimistically low, as they are dominated by the reported high confidence of studies dealing with red blood cells but may underestimate systematic errors, omissions of some cell types, and similar factors that are hard to quantify. In Fig 2, we summarize the revised results for the contribution of the different cell types to the total number of human cells. Categories contributing  $>0.4\%$  in cell count are

Box 4. Revised Estimates for the Number of Red Blood Cells, Glial

Cells, Endothelial Cells, and Dermal Fibroblasts The largest contributor to the overall number of human cells are red blood cells. Calculation of the number of red blood cells was made by taking an average blood volume of 4.9 L (SEM 1.6%, CV 9%) multiplied by a mean RBC count of  $5.0 \times 10^{12}$  cells/L (SEM 1.2%, CV 7%) (see Table 3 and S1 Data). The latter could be verified by looking at your routine complete blood count, normal values range from  $4.6\text{--}6.1 \times 10^{12}$  cells/L for males and  $4.2\text{--}5.4 \times 10^{12}$  cells/L for females. This led to a total of  $2.5 \times 10^{13}$  red blood cells (SEM 2%, CV 12%). This is similar to the earlier report of  $2.6 \times 10^{13}$  cells [1]. The number of glial cells was previously reported as  $3 \times 10^{12}$  [1]. This estimate is based on a 10:1 ratio between glial cells and neurons in the brain. This ratio of glia:neurons was held as a broadly accepted convention across the literature. However, a recent analysis [28] revisits this value and, after analyzing the variation across brain regions, concludes that the ratio is close to 1:1. The study concludes that there are  $8.5 \times 10^{10}$  glial cells (CV 11%) in the brain and a similar number of neurons and so we use these updated values here. The number of endothelial cells in the body was earlier estimated at  $2.5 \times 10^{12}$  cells (CV 40%), based on the mean surface area of one endothelial cell [1] and the total surface area of the blood vessels, based on a total capillary length of  $8 \times 10^9$  cm. We could not find a primary source for the total length of the capillary bed and thus decided to revisit this estimate. We used data regarding the percentage of the blood volume in each type of blood vessels [29]. Using mean diameters for different blood vessels [30], we were able to derive (S1 Data) the total length of each type of vessel (arteries, veins, capillaries, etc.) and its corresponding surface area. Dividing by the mean surface area of one endothelial cell [31], we derive a reduced total estimate of  $6 \times 10^{11}$  cells. The number of dermal fibroblasts was previously estimated to be  $1.85 \times 10^{12}$  [1], based on multiplying the total surface area (SA) of the human body ( $1.85 \text{ m}^2$  [32]) by the areal density of dermal fibroblasts [33]. We wished to incorporate the dermal thickness ( $d$ ) into the calculation. Dermal thickness was directly measured at 17 locations throughout the body [34], with the mean of these measurements yielding  $0.11 \pm 0.04$  cm. The dermis is composed of two main layers: papillary dermis (about 10% of the dermis thickness) and reticular dermis (the other 90%) [35]. The fibroblast density is greater in the papillary dermis, with a reported areal density,  $\sigma_{\text{pap}}$ , of  $106 \text{ cells/cm}^2$  (with  $100 \mu\text{m}$  thickness of papillary, giving  $108 \text{ cells/cm}^3$ ) [33]. The fibroblast density in the middle of the dermis was reported to be about

$3 \times 10^6$  cells/cm<sup>3</sup> [36], giving an areal density of  $\sigma_{ret.} = 3 \times 10^5$  cells/cm<sup>2</sup>. Combining these we find:  $N_{der.fib.} = SA \times (\sigma_{pap.} + \sigma_{ret.}) = 1.85 \times 10^4$  cm<sup>2</sup>  $(10^6 + 3 \times 10^5)$  cells/cm<sup>2</sup>  $= 2.6 \times 10^{10}$  cells. After this 100-fold decrease in number, dermal fibroblasts are estimated to account for only 0.05% of the human cell count.

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presented. All the other categories sum up to about 2% together. We find that the body includes only  $3 \times 10^{12}$  non-blood human cells, merely 10% of the total updated human cell count. The visualization in Fig 2 highlights that almost 90% of the cells are estimated to be nucleated cells ( $2.6 \times 10^{12}$  cells), mostly red blood cells and platelets, while the other 10% consist of  $3 \times 10^{12}$  nucleated cells. The striking dominance of the hematopoietic lineage in the cell count (90% of the total) is counterintuitive given the composition of the body by mass. This is the subject of the following analysis.

**Mass-Centered Approach as Sanity Check for Cell Count** It is prudent in making such estimates to approach the analysis from different angles. In that spirit, we now ask: does the cumulative mass of the cells counted fall within the expected range for a reference adult? To properly tackle that question, we first need to state what the anticipated result is, i.e., total body cell mass. For a reference man mass of 70 kg, 25% is extracellular fluid [37], another 7% is extracellular solids [37], thus we need to account for 4.6 kg of cell mass (including fat). A comprehensive systematic source for the composition of total cell mass (rather than total cell count) is the Report of the Task Group on Reference Man [6], which gives values for the mass of the main tissues of the human body. This mass per tissue analysis includes both intra- and extracellular components. To distinguish between intra- and extracellular portions of each tissue, we can leverage total body potassium measurements [38] as detailed in S1 Appendix. Fig 3 compares the main tissues that contribute to the human body, in terms of cell number and masses. A striking outcome of this juxtaposition is the evident discordance between contributors to total cell mass and to cell number. The cell count is dominated by red blood cells (84%), among Fig 2.

The distribution of the number of human cells by cell type. Representation as a Voronoi treemap where polygon

area is proportional to the number of cells. Visualization performed using the online tool at <http://bionic-vis.biologie.unigreifswald.de/>.

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the smallest cell types in the human body with a volume of about 100  $\mu\text{m}^3$ . In contrast, 75% of total cell mass is composed of two cell types, fat cells (adipocytes) and muscle cells (myocytes), both large cells (usually  $>10,000 \mu\text{m}^3$  by volume) that constitute only a minute fraction (0.2%) of total cell number. At the other extreme, bacteria have a minor contribution in terms of mass, but a cell count comparable to all human cells combined, as discussed above. The mass balance accounts well for all expected body mass, giving support to our analysis. The option of overlooking a collection of very small cells numerous enough to alter the total cell count is further discussed in the S1 Appendix.

**The Ratio of Bacteria to Human Cells in the Adult Body**

With the revised estimates for the number of human ( $3.0 \times 10^{13}$ ) and bacterial cells ( $3.8 \times 10^{13}$ ) in the body (the numerator and denominator of the B/H ratio), we can give an updated estimate of B/H = 1.3, with an uncertainty of 25% and a variation of 53% over the population of standard 70 kg

males. This B/H value of about 1:1 (with the associated uncertainty range) should replace the 10:1 or 100:1 values that are stated in the literature until more accurate measurements become available. We note that if one chooses to compare the number of bacteria in the human body ( $3.8 \times 10^{13}$ ) to the number of nucleated human cells ( $\approx 0.3 \times 10^{13}$ ), the ratio will be about 10:1. This is because the dominant population of non-nucleated red blood cells is not included in the calculation. We note that this ratio is the result of both the number of bacteria and the number of nucleated human cells in the body being several times lower than in the original 1970 estimate (that did not restrict the analysis to nucleated cells). The issue of whether cells without a nucleus should be included or discarded in the calculation of the number of human cells, and thus the B/H ratio, seem to be a question of definition. We view red blood cells as bona fide cells, as their names suggests. But it is also plausible to choose not to include the mass as one may think of them as “bags full of hemoglobin.” Inclusion of platelets in the count, which corresponds to their inclusion in previous counts, is also potentially disputable but has only a minor quantitative effect. Indeed, this opens an interesting tangential discussion on what should be defined as a cell. Variations in the Ratio of Bacteria to Human Cells across Population Segments After reviewing the B/H ratio for the “reference man,” we now generalize our results by addressing other segments of the population. Looking at our estimate, we identify four main parameters that dominate the calculation:

Fig 3.

Distribution of cell number and mass for different cell types in the human body (for a 70 kg adult man). The upper bar displays the number of cells, while the lower bar displays the contribution from each of the main cell types comprising the overall cellular body mass (not including extracellular mass that adds another  $\approx 24$  kg). For comparison, the contribution of bacteria is shown on the right, amounting to only 0.2 kg, which is about 0.3% of the body weight.

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1. colon volume
2. bacterial density in the colon
3. blood volume
4. hematocrit (i.e., red blood cells per unit volume).

These are the governing parameters due to the dominating contribution of the colonic bacteria and RBC count to the total bacterial and human cell counts, respectively. In order to evaluate the effect of gender, age, and obesity on the B/H ratio, we examine the change in these parameters across those groups. Table 3 collects the changes to each of the previously mentioned parameters for individuals that represent different segments of the human population: reference adult woman (1.63 m, 60 kg [39]), young infant (age 4 weeks), infant (age 1 year), elder (66 years), and obese (140 kg). Review of the literature shows no significant effect on the colonic bacterial concentrations over age from the one-month-old infant to the elderly [40,41]. The colonization of the neonatal GI tract from negligible colon bacterial concentrations of  $\approx 10^5$  bacteria/mL to concentrations equivalent to those of adults occurs in just under one month [42]. For this dynamic period that is yet to be charted in high resolution, we do not supply a B/H ratio estimate. As with age, extremes of weight have low impact on bacterial cell counts. [43]. The reported values for infants and obese are in the range of variation of “the reference man.” In addition, we could not find

any report in the literature on gender-specific differences in bacteria density in the colon. As can be appreciated from Table 3, the B/H ratio varies by up to 2-fold across those different population groups from a low of 1.3 to a high of 2.3. We note that additional factors such as race and ethnicity may influence the B:H ratio. It has been shown that the bacterial population in the colon is strongly affected by geography [47], but current data is not enough to allow robust inference for the colonic concentrations and represents a data gap.

Discussion In this study, beyond providing up-to-date estimates on the average values of the number of cells, we aimed to give representative uncertainty ranges and the variation across population

Table 3. B/H ratio for different population. See Table Bin S1 Appendix for full references.

population segment body weight [kg] age [y] blood volume [L] RBC count [ $10^{12}/L$ ] colon content [g]

bac. conc. [ $10^{11}/g$  wet] (1)

total human cells [ $10^{12}$ ] (2)

total bacteria [ $10^{12}$ ]

B:H

ref. man 70 20–30 4.9 5.0 420 0.92 30 38 1.3 ref. woman 63 3.9 4.5 480 0.92 21 44 2.2  
young infant 4.4 4 weeks 0.4 3.8 48 0.92 1.9 4.4 2.3 infant 9.6 1 0.8 4.5 80 0.92 4 7 1.7 elder  
70 66 3.8 (3) 4.8 420 0.92 22 38 1.8 obese 140 6.7 5.0 (4) 610 (5) 0.92 40 56 1.4

(1) No significant change in bacteria concentrations in relation to high variation for the reference man [40,43]. (2) Assuming RBCs account for 84% of the total host cells as observed for the reference man. (3) Decrease of 24% in the blood volume, according to [44]. (4) No significant change in the hematocrit in obesity [45]. (5) We could not find any direct measurements of the colonic volume for obese individuals in the literature, yet from an indirect analysis the volume increases with weight and plateaus at about 600 mL [46].  
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segments. This is based on comparing independent studies and the variation observed within studies. The

biggest knowledge gap we find is how realistic is the usage of the measured fecal bacteria density to represent also the average bacterial density in the colon. There is an inevitable gradient in bacterial concentration along the colon itself, from the low concentration stransiting from the ileum to the cecum of about  $10^8$  bacteria/g to  $\sim 10^{11}$  bacteria/g measured in stool. The change in bacterial concentration arises from several factors, including water absorption that concentrates the bacteria in the colon, as well as from bacterial growth during the 1–2 day transit time and the shedding of bacteria from the mucosal surface. In some respects, the estimate we performed of multiplying observed fecal bacterial density with colon content volume can be considered an upper limit. More information on the relation between the actual densities of bacteria throughout the colon and those densities measured in feces will be a big step forward in improving the estimate of this study. Another element of uncertainty is the limited information on the volume of the colon content across individuals and conditions. These knowledge gaps indicate that there might be systematic errors beyond what we could account for in the uncertainty ranges we report. In analyzing various population segments, our paper is clearly limited in scope. We touched on the obese, neonate, and elderly as well as the effect of gender but have not dealt with many other segments of interest, such as individuals in the course of antibiotic treatment or bowel preparation for colonoscopy, people with infections, chronic diseases of the GI tract, etc.

While we analyzed cell numbers, many researchers are interested in the number of genes as a reflection, for example, of the diversity of the microbiome metabolic capabilities. In order to properly estimate by what factor the genes in the bacteria we harbor outnumber our own twenty thousand genes, the very delicate question of what should be considered different genes must be properly defined, which is beyond the scope of this study. We note in passing that the number of endosymbiotic bacteria that we harbor in the form of mitochondria probably outnumbers the body bacteria several fold. This can be appreciated by noting that most cell types (though not red blood cells) contain hundreds (or more) of mitochondria per cell [48].

Should we care about the absolute number of human cells in the body or the ratio of bacterial to human cells? Updating the ratio of bacteria to human cells from 10:1 or 100:1 to close to 1:1 does not take away from the biological importance of the microbiota. Yet, we are convinced that a number widely stated should be based on the best available data, serving to keep the quantitative biological discourse rigorous. The study of absolute numbers is also of relevance for specific biological questions. For example, a recent study showed how knowing the number of cells in different tissues

can be an important indicator in understanding variation in cancer risk among tissues [49]. Other applications refer to the dynamic processes of development and mutation accumulation. Finally, the type of numeric focus exercised here reveals and attracts attention to knowledge gaps such as the bacterial population densities in the proximal colon and how well are they represented by current analysis methods. We thus became aware through this study of promising steps forward in fulfilling the Delphic maxim of “know thyself” from a quantitative perspective.

Supporting Information S1 Appendix. Supplementary information text. Elaboration of calculation methods and sanity check. (DOCX)

S1 Data. Detailed calculations. Spreadsheets with the detailed calculations mentioned throughout the text and references for all data sources. (XLSX)

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Oral manifestation in inflammatory bowel disease: A review

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Abstract Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis, not only affect the intestinal tract but also have an extraintestinal involvement within the oral cavity. These oral manifestations may assist in the diagnosis and the monitoring of disease activity, whilst ignoring them may lead to an inaccurate diagnosis and useless and expensive workups. Indurated tag-like lesions, cobblestoning, and mucogingivitis are the most common specific oral findings encountered in CD cases. Aphthous stomatitis and pyostomatitis vegetans are among non-specific oral manifestations of IBD. In differential diagnosis, side effects of drugs, infections, nutritional deficiencies, and other inflammatory conditions should also be considered. Treatment usually involves managing the underlying intestinal disease. In severe cases with local symptoms, topical and/or systemic steroids and immunosuppressive drugs might be used. © 2013 Baishideng Publishing Group Co., Limited. All rights reserved.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Extra-intestinal manifestations; Pyostomatitis vegetans; Aphthous stomatitis; Cobblestoning; Mucogingivitis; Oral manifestation

Core tip: Although the gastrointestinal tract is the primary site of involvement in inflammatory bowel disease (IBD) patients, some cases might present with nonintestinal manifestations, such as oral lesions. These oral manifestations may aid in the diagnosis and the monitoring of disease activity, whilst ignoring them may lead to an inaccurate diagnosis and useless and expensive workups. Indurated tag-like lesions, cobblestoning, mucogingivitis, aphthous stomatitis, and pyostomatitis vegetans are the main oral presentations of IBDs. With the growing incidence of IBDs and the increased likelihood of encountering these particular manifestations, this review summarizes various oral findings seen in IBD cases by describing their unique morphologic description, treatment recommendations, and probable differential diagnosis.

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INTRODUCTION Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases with primary intestinal involvement[1-5]. Although the exact underlying pathogenesis of IBD has not been clearly elucidated, it is postulated that dysregulated immunity is its basis[4,6-12]. Generally, it is assumed that IBD is a multifactorial disease in which immune system, genetics, and environmental factors all have a

REVIEW

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role[2,8,13-17]. Other than the expected symptoms of gastrointestinal involvement, IBD patients may exhibit a wide range of non-intestinal signs and symptoms known as extraintestinal manifestations (EIMs), with prevalence rates ranging from 6%-47%[2,8,18,19]. Approximately one third of IBD patients develop EIMs in the course of their disease[1,20-25]. Joints, skin, eyes, and the biliary tract are among the most common organs involved in EIMs[22,26-28]. Oral involvement with different presentations may also be seen in IBD. Oral manifestations could also occur in these patients due to other causes, such as drug reactions, infections, and unrelated diseases[1,2,6,8,20,21]. Patients with IBD may present with these oral manifestations years before the appearance of intestinal disease[1,6]. Recognizing these patterns may assist physicians and other care givers in making a timely diagnosis of IBD while avoiding unnecessary workups[29]. The scope of this review is to describe various oral presentations in IBD and their differential diagnosis and treatment.

**EPIDEMIOLOGY OF ORAL MANIFESTATIONS IN IBD** In 1969, Dyes and colleagues initially described oral lesions in two patients with CD[7,30]. This was followed in the same year by Dudeney's report of another patient suffering from CD who had oral manifestation[31]. Oral lesions in the absence of intestinal findings in CD were initially described in 1972 by Varley[32], and there have since been various reports on the incidence of oral lesions in CD[1-3,23,30,33-39]. The highest rate was reported from a study in a pediatric age group, indicating a rate of 48%[33]. The prevalence rate is estimated to be between 50% and 20% in most publications[1,33,38]. This variation in rate might be related to the different ages of patients studied, their ethnicity and genetic background, whether they were receiving treatment while being investigated, the experience level of the examiners, and the variation in definition of specific lesions[34]. In the majority of cases, intestinal involvement precedes the oral lesion[1]. Oral lesions are more common in CD compared to UC, more prevalent in children compared to adults, and with a male dominance[1,8,21,33,34,37,40,41]. The prevalence is also higher in CD patients with proximal gastrointestinal tract and/or perianal involvement[2,33,42]. Oral lesions may be the primary presenting signs preceding gastrointestinal symptoms[43,44] in 5%-10% of affected patients[39]. This figure has been reported to be as high as 60% in patients with CD[37]. Although the lesions might be more severe at the time of active disease, the correlation is not universal, and up to 30% of affected patients may continue to manifest oral lesions (especially in the pediatric age group) despite disease control[34,45].

**ORAL LESIONS IN CROHN'S DISEASE** Dudeney's report of oral Crohn's disease in 1969 described it as a raised, edematous, pink granulation tissue in the buccal mucosa[31]. It is now known that the lips are the most frequent site of oral Crohn's disease (OCD) lesions[37]. Oral lesions may be painful, impair proper oral function, or lead to psychological disorders due to disfigurement[8,46]. Oral manifestations of CD can be specific or non-specific, based on the presence of granulomas noted on the histopathology reports[1].

**SPECIFIC ORAL CROHN'S DISEASE LESIONS** These specific lesions contain granulomatous changes noted upon histopathological examination. They are less common than non-specific lesions, and can occur either concomitantly with intestinal symptoms or before gut

presentation by several years[47,48]. The most affected portions in the mouth are the buccal mucosa, gingiva, lips, vestibular, and retromolar areas[32]. There are four main lesions, as described below and shown in Table 1.

**INDURATED TAG-LIKE LESIONS** These are white reticular tags[35] referred to as mucosal tags, epithelial tags, or folds[49]. These lesions are mostly discovered in the labial and buccal vestibules, and in the retromolar regions[21]. Up to 75% of these lesions may show non-caseating granulomas on histopathology[33,42]. There has been no specific direct association of these lesions with intestinal CD activity reported[1]. Treatment is described in the later section on general treatments of OCD lesions.

**COBBLESTONING** Fissured swollen buccal mucosa with corrugation and hyperplastic appearance of the mucosa are called cobblestoning[1,42,50,51]. These lesions are usually seen in the posterior buccal mucosa and may be associated with succulent mucosal folds with normal epithelium[21]. The lesions usually consist of mucosal-colored papules that produce firm plaques on the buccal mucosa and palate. Such lesions may cause pain and make speaking and eating normally difficult[52]. These lesions, along with mucosal tags, are considered pathognomonic for CD[35], but are not associated with intestinal CD activity[1]. Treatment consists of topical steroids in addition to the treatment of intestinal involvement. In more severe presentations, systemic steroids could be used[53].

**MUCOGINGIVITIS** The gingiva may become edematous, granular, and hyperplastic in Crohn's disease, with or without ulceration. The whole gingiva up to the mucogingival line might be involved[7,30]. As with other specific lesions of the oral cavity, this lesion has no association with intestinal CD activity. Treatment is discussed in the section on general

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treatments of OCD lesions below.

**OTHER SPECIFIC LESIONS** Lip swelling with vertical fissures, deep linear ulcerations (usually in the buccal sulci with hyperplastic folds), and midline lip fissuring may also occur in CD[1,2,7,8,22,30,33,35,39,42, 49,54]. These lesions also have no association with intestinal CD activity[1]. While these lesions may be very incommodious for patients, they can be treated with topical tacrolimus at low concentration (0.5 mg/kg) and intra-lesional steroid injection with or without mandibular blockade[34,55,56]. In more severe cases with persistent pain and cosmetic disfigurement, more aggressive therapy with immunosuppressive agents is recommended[34].

**NON-SPECIFIC ORAL LESIONS IN CD** Table 1 provides details of various non-specific oral lesions that occur with Crohn's disease.

**APHTHOUS STOMATITIS** Aphthae are shallow round ulcerations with central fibrinous exudate and an erythematous border[23,57]. These lesions may occur in 20%-25% of the general population[3,58], up to 10% of patients with UC, and 20%-30% of those with CD that have oral aphthosis[4]. In a survey conducted in Iran, oral aphthous lesions were found in approximately 13% of CD vs 6% of UC patients[13]. The association of oral aphthosis and disease activity is not clear. While it may become more severe in active disease, its presence does not correlate with disease activity. Patients with IBD and other EIMs may suffer recurrent aphthous stomatitis more often than others[4]. Aphthous stomatitis has been associated with ankylosing spondylitis, uveitis, peripheral arthritis, and erythema nodosum[59]. Aphthous eruptions are not specific for IBD and may be observed in several other disorders including celiac sprue, HIV/AIDS, Behçet's disease, and Reiter's

syndrome, as well as common aphthae seen in the normal population[23,60-66]. Management of CD is usually sufficient for control of oral aphthosis. For control of pain, topical agents (such as lidocaine) and/or topical steroids (such as triamcinolone 0.1%) up to three times per day can be used. Dexamethasone elixir (0.5 mg/5 mL spit or swish) or ointment up to three times per day is also efficacious. Non-steroidal anti-inflammatory pastes are effective in

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Table 1 Summary of specific and non-specific oral lesions in Crohn's disease

Lesion Relation with CD activity Frequency Treatment options

Specific oral lesions

Indurated tag-like lesions No specific direct association reported

Common in OCD patients See general points on the treatment of OCD in the text

Cobblestoning No specific direct association reported

Common in OCD patients Topical steroids for less severe cases and systemic steroids for others

Mucogingivitis No specific direct association reported

Common in OCD patients See general points on the treatment of OCD in the text Others: Lip

swelling with vertical fissures No specific direct association reported Topical tacrolimus,

intra-lesional injection of steroids, immunosuppressive agents Deep linear ulcerations

Topical analgesics, 5-ASA, or steroids, intra-lesional steroids, topical tacrolimus, other

medications used in PV treatment

Non-specific oral lesions

Aphthous stomatitis No specific direct association reported

10% of patients with UC and 20%-30% of those with CD

Topical agents (lidocaine 2%, triamcinolone 0.1%, dexamethasone elixir), non-steroidal antiinflammatory pastes, systemic steroids, intralesional steroids

Pyostomatitis vegetans Associated with active CD

Rare Antiseptic mouthwashes/topical steroids (though less effective), systemic steroids, azathioprine and sulfamethoxypyridazine, dapsone, cyclosporine A, injections of infliximab pursued by maintenance therapy with MTX, adalimumab, surgical colectomy in UC

Others: Angular cheilitis No specific direct association reported

Unknown 5-ASA mouthwashes, topical steroids (1% hydrocortisone), vitamin supplements, intra-lesional steroids

Persistent submandibular lymphadenopathy

See general points on the treatment of OCD in the text Recurrent buccal abscesses

Antibiotics, infliximab, methotrexate, thalidomide Perioral erythema with scaling Glossitis

CD: Crohn's disease; OCD: Oral Crohn's disease; MTX: Methotrexate; UC: Ulcerative colitis.

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roduced by McCarthy in 1949[38], but its association with IBD was not initially

recognized[38]. PV is a chronic mucocutaneous ulcerative disorder consisting of multiple

miliary white or yellow pustules with an erythematous and edematous mucosal

base[1,23,77,79]. The pustules can rupture and coalesce to form linear or "snail-track"

ulcers[1,23,38,77,78,80]. The most frequently involved regions of the oral cavity are the

labial gingiva, labial, and buccal mucosa[78]. The least damaged portions are the tongue

and floor of the mouth[1], but pustules can involve almost all parts of the oral cavity[78].

PV can be seen at any age, but is more prevalent in patients aged between 20 and 59 years, with an average age of 34 years. Males are affected more frequently than females, with a ratio of 2:1-3:1[81,82]. PV is considered to be the oral equivalent of pyodermatitis vegetans on the skin[77,78]. There is a strong association between PV and IBD, and the former is a specific marker of disease activity in UC[1,2,38,39,78,83,84]. Intestinal involvement usually predates the onset of PV in IBD, although this could be asymptomatic and mild[23,85]. Despite every effort, no bacterial, fungal, or viral cause has yet been found for this manifestation and its pathogenesis remains obscure[77]. The principal histological features on microscopy are intra-epithelial and/or sub-epithelial micro-abscesses with neutrophils and eosinophils. Furthermore, hyperkeratosis, acanthosis, and acantholysis are seen in histology examination[1,38,40,78,86]. Direct immunofluorescence in PV is negative for deposits of IgA, IgG and C3 and this result is helpful in distinguishing PV from pemphigus vulgaris[1,87]. Clinically, the patient may have fever, enlarged and tender submandibular lymph nodes, and pain. Pain intensity is variable; some patients with extensive oral lesions may not have any pain[78]. Peripheral eosinophilia is seen in up to 90% of cases and is the main laboratory finding in many patients[87]. The diagnosis of PV is based on a constellation of clinical features that include concurrent IBD, peripheral eosinophilia, histological study, and negative culture results of the lesion exudate. As mentioned above, a negative immunofluorescence study is also helpful[1,77,78]. The main differential diagnoses of PV include vesicular disorders involving both the skin and oral cavity; similar to pemphigus vulgaris in particular, as well as other diseases like bullous pemphigoid, acquired epidermolysis bullosa, bullous drug eruptions, herpetic infection, Behçet's disease, and erythema multiforme[1,77,80,88]. Herpetic infections should be excluded by Tzanck smear, antigen detection, and culture of the virus, or PCR for HSV virus[23]. The mainstay in the management of PV is the treatment of underlying IBD. Topical steroids and antiseptic mouthwashes alone are effective in only a few instances. Systemic steroids are usually prescribed for these patients and are considered as the treatment of choice. Azathioprine and sulfamethoxypyridazine can be used in parallel with steroids as sparing agents[3,21,23,38,77,78]. Dapsone is another option, but should be used as a second line agent, especially in relapsing cases. Hemolytic ane relieving pain and promoting healing. Systemic or intralesional steroids should be reserved for severe refractory and/or persistent cases[4,13,21,32,67-70].

**PYOSTOMATITIS VEGETANS AND OTHER NON-SPECIFIC LESIONS** Pyostomatitis vegetans can occur in both UC and CD, but is more common in the former and will be discussed in more detail in the later section addressing oral manifestations of UC. Other non-specific oral findings of CD include angular cheilitis, persistent submandibular lymphadenopathy, sicca syndrome and reduced salivation, halitosis, dental caries and periodontal involvement, candidiasis, odynophagia, dysphagia, minor salivary gland enlargement, perioral erythema with scaling, recurrent buccal abscesses, glossitis, mucosal discoloration, lichen planus, and metallic dysgeusia[2,7,21,32,34,35,40,54,71]. For the management of angular cheilitis, 5-ASA mouthwashes, topical steroids (1% hydrocortisone), vitamin supplements, and intra-lesional steroids may be effective. Antibiotics are the first step in treating recurrent buccal abscesses. For more severe cases, immunomodulating agents including chimeric antitissue necrosis factor (TNF) alpha monoclonal antibodyinfluximab, methotrexate, and thalidomide have been used[7,21].

**GENERAL POINTS ON THE TREATMENT OF OCD** In the majority of patients with OCD, the oral findings are asymptomatic and clinically silent. In these patients, no peculiar treatment is needed for oral lesions and the latter will resolve over time in association with the treatment of gastrointestinal disease using anti-inflammatory drugs (5-ASA), immunosuppressive agents, and finally biological agents, whichever are indicated[8,21,34,40,72]. The treatment armamentarium includes topical and systemic steroids, 5-ASA compounds, immunosuppressive agents, biologic treatments, and even antibiotics such as tetracycline[2,73]. The first and foremost step in treating oral lesions is to control colonic disease[74]. Food restriction, which is discussed later in the management of orofacial granulomatosis (OFG), could also be tried in OCD[75,76].

**ORAL LESIONS IN UC** There are many similarities between the oral manifestations of CD and UC. Although oral lesions are more common in CD, almost all of the so-called non-specific oral lesions described in CD can also occur in UC. Among these lesions, pyostomatitis vegetans occurs more commonly in UC than in CD and will be discussed here in more detail[1,2,74,77,78]. The term pyostomatitis vegetans (PV) was first in Lankarani KB et al . Oral manifestations of IBDs

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mia, hepatitis, agranulocytosis, and drug-induced allergic reactions are the major side effects of dapsone requiring attention[3,78]. Calcineurin blockers like cyclosporine A have been successfully used, as described in case reports in the treatment of PV[89]. Injections of infliximab followed by maintenance therapy with methotrexate have been also effective, especially in PV associated with CD[77]. Systemic use of newer humanized anti-TNF agents like adalimumab has also proven effective in inducing remission of both oral and gastrointestinal manifestations[77]. Surgical colectomy produces promising results in PV associated with UC[3,78,90]. Other non-specific findings in UC include oral aphthae, glossitis, cheilitis, stomatitis, lichen planus, mucosal ulcers, diffuse pustules, and non-specific gingivitis[1-3,23,42]. In a report of patients with UC, 4.3% had aphthous stomatitis during intestinal disease flare-ups[2], thus the presence of this non-specific manifestation may have some correlation with disease activity in UC.

**DIFFERENTIAL DIAGNOSES** Because CD is a granulomatous disorder, all other diseases capable of inducing granulomatous reaction in the oral cavity are included in the differential diagnosis (DDX) list. The most common cause of developing oral granulomas is a response to foreign bodies, primarily dental materials such as retained amalgams or endodontic sealers[91]. The second important DDX to be considered is tuberculosis bacilli. Special staining processes for acidfast bacilli, PPD skin test, sputum culture, and chest X-ray are often used to diagnose oral tuberculosis[2,80,92]. Fungal infections such as candidiasis, histoplasmosis, cryptococcosis, paracoccidioidomycosis, and blastomycosis can all trigger granulomatous involvement of the mouth. The presence of these infections could be confirmed by special stains including applying PAS or Gömöri trichrome stain and, more specifically, with molecular studies[2,21,80]. Oral sarcoidosis should always be considered in DDX, and an appropriate workup should include measuring serum angiotensin converting enzyme, IL-2 receptor level, IL-8 level, and chest X-ray in suspected cases[2,6,21,39,93]. Leprosy, cat scratch disease, tertiary syphilis, orofacial granulomatosis, T-cell lymphoma, and Wegener's disease can all produce a granulomatous reaction in the oral cavity, but are much rarer and usually have other prominent features leading to diagnosis[21,39]. Considering the role of nutritional deficiencies is of utmost importance as

stomatitis, glossitis, aphthous ulcers, cheilitis, or perioral dermatitis may occur with nutrient deficiencies resulting from an insufficient supply of the vitamin B family, albumin, iron, folate, zinc, niacin, and/or other essential elements[8,41,94-97]. Nutrient deficiencies may be the result of intestinal involvement or may be caused by the medications used in the treatment of

IBD[98,99]. Sulfasalazine and azathioprine, for instance, may cause folate and niacin deficiency, respectively[2]. Other non-specific oral manifestations may also be related to the side effects of drugs. As an example, oral aphthosis has been reported in association with nonsteroidal anti-inflammatory agents, nicorandil[100], and bupropion[101]; gingival hyperplasia with cyclosporine[102], amlodipine[103], and anticonvulsants such as phenytoin[104]; and reversible lichen planus with sulfasalazine[54].

**OROFACIAL GRANULOMATOSIS** Gibson et al[40] used the term OFG in 1985 to define a constellation of oral signs similar to those seen in OCD, but in the absence of evidence of intestinal CD. In this rare syndrome, chronic swelling of the lips and lower half of the face is prominent, in association with oral ulcers and hyperplastic gingivitis. Granulomatous cheilitis is the most common sign seen in OFG[105]. The most frequent sites of involvement in OFG are the lips, which may be individually or both involved[80]. Lip swelling usually leads to painful vertical fissures[2]. Three forms of ulcers are found in OFG: deep buccal ulcers with raised peripheral mucosa, aphthous-type ulcers, and micro-abscesses located commonly on the gingival margin or soft palate[21]. In general, the ulcers are mainly superficial and the gingivae are erythematous with patchy distribution, mostly affecting the anterior portion. These alterations extend from the free gingival margin to the non-keratinized mucosa of the sulci, developing a full-thickness gingivitis pattern[40]. In the largest series of studies involving OFG reported to date, the mean age of those affected at presentation was 20 years with no gender predilection. With the pathogenesis unknown, allergic, infectious, and genetic causes have also been postulated[40,106]. Unlike OCD in which Th1 CD4+ lymphocytes are the dominant population, in OFG the overstimulation of Th2 CD4+ lymphocytes is detected in biopsy specimens, where it is shown as infiltrating cells[21]. Granulomas noted upon histology examination are the hallmark in both OFG and OCD. The only way to exclude CD is by clinical presentation[21]. As mentioned previously, oral manifestations may precede gastrointestinal involvement in CD for many years. Thus, cases labeled as OFG may later progress to being diagnosed as CD[21,34]. Recently, it has been reported that 4 out of 6 children with OFG in early childhood were reported as having developed CD on follow-up[34]. A rare presentation of OFG seen in adults is Melkersson-Rosenthal syndrome; a triad of orofacial swelling, intermittent facial paralysis, and a fissured tongue[21,34,107]. Observational studies in pediatric patients with OFG have demonstrated that dietary elimination of some triggering elements (encompassing cinnamaldehyde, benzoate additives, carnosine, monosodium glutamate, cocoa, and sunset yellow) are effective in the treatment of oral lesions[75,76]. Analgesia and topical agents like beclomethasone mouthwash and 5-ASA spray or ointments can be

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used as basic therapies. In unresponsive cases, treatment with systemic steroids and immunosuppressive medications can be used[21]. Clofazimine, a drug used in the treatment of leprosy, is occasionally effective in OFG[37].

**CONCLUSION** Oral manifestations of inflammatory bowel diseases are diverse. Although they are generally more common in patients with Crohn's disease, specific manifestations like PV occur more commonly in ulcerative colitis, which is associated with disease activity in most instances. Most other manifestations have no correlation with disease activity. In differential diagnosis of these oral manifestations, side effects of drugs, nutritional deficiencies, infections, as well as other granulomatous diseases with oral involvement should all be considered. There is usually no need for specific treatment for these lesions, but when indicated it may comprise topical and systemic steroids, immunosuppressive drugs, antibiotics, and even biological treatment in more severe cases.

Good Oral Health and Diet

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Copyright © 2012 G. A. Scardina and P. Messina. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. An unhealthy diet has been implicated as a risk factor for several chronic diseases that are known to be associated with oral diseases. Studies investigating the relationship between oral diseases and diet are limited. Therefore, this study was conducted to describe the relationship between healthy eating habits and oral health status. The dentistry has an important role in the diagnosis of oral diseases correlated with diet. Consistent nutrition guidelines are essential to improve health. A poor diet was significantly associated with increased odds of oral disease. Dietary advice for the prevention of oral diseases has to be a part of routine patient education practices. Inconsistencies in dietary advice may be linked to inadequate training of professionals. Literature suggests that the nutrition training of dentists and oral health training of dietitians and nutritionists is limited.

**1. Introduction** The concept of oral health correlated to quality of life stems from the definition of health that the WHO gave in 1946. Health is understood to be "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity". The programs for the prevention of oral diseases concern teaching about oral hygiene and healthy eating, fluoride prophylaxis, periodic check-ups, sessions of professional oral hygiene, and secondary prevention programs [1]. The term "bionutrition" refers to the important interactions which exist between diet, use of nutrients, genetics, and development. This term emphasizes the role of nutrients in maintaining health and preventing pathologies at an organic, cellular, and subcellular level [2]. There exists a biunique relationship between diet and oral health: a balanced diet is correlated to a state of oral health (periodontal tissue, dental elements, quality, and quantity of saliva). Vice versa an incorrect nutritional intake correlates to a state of oral disease [3–6].

**2. Diet and the Development of the Oral Cavity**

Diet influences the development of the oral cavity: depending on whether there is an early or late nutritional imbalance, the consequences are certainly different. In fact, an early nutritional imbalance influences malformations most. Moreover, the different components of the stomatognathic apparatus undergo periods of intense growth alternated with periods of relative quiescence: it is clear that a nutritional imbalance in a very active period of growth will produce greater damage [3]. A shortage of vitamins and minerals in the phase before conception influences the development of the future embryo, influencing dental organogenesis, the growth of the maxilla, and skull/facial development [1, 2]. An insufficient supply of proteins can lead to [3, 4] the following: (i) atrophy of the lingual papillae, (ii) connective degeneration, (iii) alteration in dentinogenesis, (iv) alteration in cementogenesis, (v) altered development of the maxilla, (vi) malocclusion, (vii) linear hypoplasia of the enamel. An insufficient supply of lipids can lead to [5, 6] the following: (i) inflammatory and degenerative pathologies,

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Figure 1: Caries of the teeth.

(ii) parotid swelling—hyposalivation, (iii) degeneration of glandular parenchyma, (iv) altered mucosal trophism.

An insufficient supply of carbohydrates can lead to the following:

(i) altered organogenesis, (ii) influence of the metabolism on the dental plaque, (iii) caries, (iv) periodontal disease.

Diet influences the health of the oral cavity, conditioning the onset of caries, the development of the enamel, the onset of dental erosion, the state of periodontal health, and of the oral mucous in general.

### 3. Caries

Caries is a demineralization of the inorganic part of the tooth with the dissolution of the organic substance due to a multifactorial aetiology. The demineralization of the enamel and of the dentine is caused by organic acids that form in the dental plaque because of bacterial activity, through the anaerobic metabolism of sugars found in the diet [7]. Demineralization occurs when the organic acids produced increase the solubility of the calcium hydroxyapatite that is present in the hard tissue of teeth (Figure 1).

The development of caries requires the presence of sugars and bacteria but it is influenced by the susceptibility of the teeth, by the type of bacteria, and by the quantity and quality of the salivary secretion. Saliva is supersaturated with calcium and phosphate with a pH equal to 7, a level that favours remineralization. When acid stimulation is too strong demineralization prevails until the formation of a carious lesion [8].

Very low levels of dental caries are found in isolated communities with a traditional lifestyle and low consumption of sugars [7–9]. As soon as economic conditions improve and the quantity of sugars and other fermentable carbohydrates increases in the diet, a notable increase in dental caries is noticed. This has been seen in the Inuit of Alaska and in populations in Ethiopia, Ghana, Nigeria, Sudan, and the islands of Tristan da Cunha and Sant'Elena [7–9]. A Vipeholm study in Sweden between 1945 and 1953 in an institute for the mentally ill underlined the correlation between caries and the intake of sugary food of variable viscosity. If the sugar was ingested up to a maximum of 4 times a day only during meals, it had little effect on the increase of caries, even if this occurred in great quantities;

the increase in the frequency of consumption of sugar between meals was associated to an increase in caries; when they no longer ate foods rich in sugar, the incidence in the formation of caries diminished [10]. The types of sugar ingested through diet also influence the onset of illness. In fact, studies on the pH of the dental plaque have shown that lactose produces less acidity in comparison to other sugars. A 1970 Finnish study on a supervised dietary change revealed that, in an adult population, the almost total substitution of sucrose in the diet with xylitol determines a 85% reduction in caries over a 2-year period; its mechanism of action resides in the inhibition of the growth of *Streptococcus mutans*, the most important microorganism responsible for the formation of caries [11]. Diet can be a good ally in the prevention of caries [12].

(i) Increase in the consumption of fibres: diminution of the absorption of sugars contained in other food. (ii) Diets characterized by a ratio of many amides/little sugar have very low levels of caries. (iii) Cheese has cariostatic properties. (iv) Calcium, phosphorus and casein contained in cow milk inhibit caries. (v) Wholemeal foods have protective properties: they require more mastication, thus stimulating salivary secretion. (vi) Peanuts, hard cheeses, and chewing gum are good gustative/mechanical stimulators of salivary secretion. (vii) Black tea extract increases the concentration of fluorine in the plaque and reduces the cariogenicity of a diet rich in sugars. (viii) Fluorine.

Fluorine remains a milestone in the prevention and in the control of dental caries. It has a preeruptive mechanism of action (incorporation in the enamel during amelogenesis) and a posteruptive mechanism (topical action). Fluorine reduces caries by 20–40% in children, but it does not entirely eliminate them: even when fluorine is used, the association between the intake of sugars and caries continues to be present all the same [13]. Diet also influences the qualitative characteristics of salivary secretion. These secretive proteins (mucines) represent an important barrier against the reduction of humidity, against the physical and chemical penetration of irritants and against bacteria [14].

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Figure 2: Hypoplasia and pits on the surface of the enamel correlate to a lack of vitamin A.

Figure 3: Hypoplasia on the surface of the enamel correlate to a lack of vitamin D.

The synthesis of glycoproteins requires vitamin A. In an imbalanced diet, there is a reduction in the content of mucines with the consequent risks for oral health (Caries!!).

#### 4. Development of the Enamel

Teeth in a preeruptive phase are influenced by the nutritional state. A lack of vitamins D and A and protein-energy malnutrition have been associated to hypoplasia of the enamel and atrophy of the salivary glands, conditions that determine a greater susceptibility to caries. Some hypoplasia and pits on the surface of the enamel correlate to a lack of vitamin A (Figure 2); a lack of vitamin D is associated to the more diffused hypoplastic forms (Figure 3). The structural damage can testify to the period in which the lack of nutrition occurred [15].

#### 5. Dental Erosion

“Dental erosion is the progressive irreversible loss of dental tissue that is chemically corroded by extrinsic and intrinsic acids through a process that does not involve bacteria...”

Extrinsic Acids Derived from Diet. They citric, phosphoric, ascorbic, malic, tartaric, and carbonic acids that are found in fruit, in fruit juices, in drinks, and in vinegar.

Figure 4: Dental Erosion.

Figure 5: Periodontal disease.

Intrinsic Acids. They are derived from serious gastroesophageal reflux [16–18](Figure 4).

#### 6. Periodontal Disease

Periodontal disease evolves more quickly in undernourished populations: “...the pathology starts in the gum and could interest the periodontal ligament up to the alveolar bone...”.

The most important risk factor in the development of periodontal disease is represented by inadequate oral hygiene (Figure 5). Data supplied by the National Health and Nutrition Examination Survey 2001/02 underlined that a low level of folic acid is associated to periodontal disease. The serum level of folates is an important index of periodontal disease and can represent an objective that should be pursued in the promotion of periodontal health [19]. Malnutrition and bad oral hygiene represent two important factors that predispose for necrotizing gingivitis. Prevention programs against disease must therefore include a correct evaluation of the immune system and the promotion of nutritional programs. The aim of nutritional support in inflammatory diseases is to provide the right energy and

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Cyclopia (B2)

Holoprosencephaly (B1)

Hemicephalus (B3)

Median cleft (7)

Naso-ocular cleft (2) Unilateral cleft (1)

Horizontal cleft (5) Medial oro-ocular cleft (3) 5 wks. ?  
?

Lower midline cleft (6)

Treacher Collins syndrome

Lateral oro-ocular cleft (4)

Figure 6: Cleft lip and palate.

nourishment to respond to the increased demand for protein synthesis in the acute phase, inflammatory mediators, antioxidant defence mechanisms, as well as for the promotion of tissue reparation. Some nutrients have a very important role in the resolution of the inflammatory process. These observations confirm the relationship between diet and periodontal disease [20]. In a recent interview, the president of the American Society of Periodontology, Michael P. Rethman [20], underlined the importance of diet for a healthy smile. In particular, the correlation between the intake of calcium and periodontal disease can be due to the role that calcium has in the density of the alveolar bone that supports teeth. Also the intake of vitamin C is fundamental for maintenance and for the activation of reparative mechanisms thanks to its antioxidant properties [20]. Noma is an orofacial gangrene originating in the gingival-oral mucosa [21]. Although cases of noma are now rarely reported in the developed countries, it is still prevalent among children in third world countries, notably

in subSahara Africa, where malnutrition and preventable childhood infections are still common [21]. Noma can be prevented through promotion of national awareness of the disease, poverty reduction, improved nutrition, promotion of exclusive breastfeeding in the first 3–6 months of life, optimum prenatal care, and timely immunisations against the common childhood diseases [21].

## 7. Gene Disease

Italian researchers have recently identified the genetic defect responsible for cleft lip and palate (Figure 6). The gene is a variation of the maternal gene "MTHFR" that determines the lowering of folate levels in blood. The female carriers of the discovered mutation have a greater risk of giving birth to children affected by cleft lip and palate. Folate is fundamental in the first phases of embryonic development: in fact the lack of this vitamin is able to cause defects in the embryonic development known generically as "defects of the neural tube".

For this reason, in the United States B9 is

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Figure 7: Oral cancer.

Figure 8: Oral cancer.

administered with the support of the health authorities to women who are intend to conceive and in the first months of pregnancy. Administering folate in the months preceding conception and in the first months of pregnancy, the risk of defectstothenervoussystemisreducedandeven cleftlipand palatecouldbeavoidedwiththepreventiveadministrationof the vitamin [22].

8. Neonatal Diet and Oral Health The World Health Organization and the American Pediatric Association have shown that breastfeeding influences lingual deglutition, the growth of the maxillae and the correct alignment of the teeth, as well as the modelling of the hard palate. Vice versa, bottle feeding the baby influences the formation of the ogival palate as well as the formation of "crossbite", a reduced opening of the back nasal cavity, and an increased incidence of sleep apnea. In addition, artificial feeding influences the possibility of the onset of arterial hypertension, obesity, cardiovascular illnesses, and inflammatory pathologies regarding oral mucous [23, 24].

9. Oral Cancer The association between diet and oral cancer is extremely serious. It is a pathology that is diagnosed in three hundred

Figure 9: Oral cancer.

thousand new cases in the world every year and presents the greatest incidence in people who smoke, chew tobacco, and consume alcohol (Figures 7, 8, and 9). The use of tobacco can alter the distribution of nutrients such as antioxidants, which develop a protective action toward the cells: smokers present levels of carotenoids and vitamin E in the blood that are superior to those in the oral mucous and, in addition, have a different distribution in comparison to the norm; the levels of folates in the blood and in the cells of the oral tissues of smokers are inferior to those of nonsmokers; the inside of the cheeks of smokers presents numerous micronuclei (modifications typical of pre- and neoplastic lesions) [25, 26]. The study of the incidence of this illness has underlined the possibility that diet can represent an important etiologic factor for oral carcinogenesis. Vitamins A, E, C, and Beta Carotene have antioxidant properties. (i) They neutralize metabolic products. (ii) They interfere with the activation of procarcinogens. (iii) They inhibit chromosomal aberration. (iv) They potentially inhibit the growth of malignant lesions (leukoplakia). The mechanism that connects smoke to this disease has not been discovered but some progress has been made: smoke modifies the distribution of protective substances such as folates and some antioxidants. A rebalancing of nutrients obtained through diet can modify the altered distribution caused by the consumption of tobacco. In an imbalanced diet there is a depletion of antioxidant nutrients. Fruit and vegetable have, vice versa, important antioxidant properties. Many micronutrients (vitamins

in particular) are used in chemoprevention programs formulated by the US National Cancer Institute [27]. The National Cancer Institute and the American Cancer Society have established some prudential dietary recommendations for the choice of food: (1) maintain a desirable body weight, (2) eat a varied diet, (3) include a new variety of fruits and vegetables in the daily diet,

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(4) consume a greater quantity of foods rich in fibre, (5) decrease the total intake of fats (30% less than the total calories), (6) limit the consumption of alcohol, (7) limit the consumption of salted food or food preserved with nitrates.

In patients with an advanced tumour disease, protein-caloric malnutrition is a recurrent problem due to factors such as a form of anorexia that is established, maldigestion, malabsorption, and to a difficulty in mastication and deglutition [26]. Foods should be provided that aim to correct nutritional deficits and ponderal reduction when consumed in a large enough quantity to cover protein and caloric requirements. Malnutrition also interferes negatively with humoral and cellular immunocompetence and with tissue and reparative functions. In addition, the alteration of the liver function can change the way drugs are metabolized. Therefore, malnutrition can interfere with oncological therapy and increase the severity of the collateral effects [25]. Some studies show a small effect of dietary supplementation on cancer incidence, while others show that supplementation with antioxidant vitamins may have an adverse effect on the incidence of cancer and cardiovascular diseases or no effect [27]. Increasing attention has been given to the potential protective roles of specific antioxidant nutrients found in fruits and vegetables. In a recent research El-Rouby showed that lycopene can exert protective effects against 4-nitroquinoline-1-oxide induced tongue carcinogenesis through reduction in cell proliferation and enhanced cellular adhesion, suggesting a new mechanism for the anti-invasive effect of lycopene [28]. In a recent report Edefonti et al. showed that diets rich in animal origin and animal fats are positively, and those rich in fruit and vegetables and vegetable fats inversely related to oral and pharyngeal cancer risk [29].

#### 10. Oral Candidosis

A significant correlation has been evinced with a lack of iron (Figure 10). This causes alterations in the epithelium with consequent atrophy and alteration in cellular turnover, an alteration in the iron-dependent enzymatic system depression in cell-mediated immunity, phagocytosis, and in the production of antibodies. The correlation between candidiasis and the lack of folic acid, vitamins A, B1, B2, vitamins C, K, zinc, and a diet rich in carbohydrates is also significant [30].

11. Potentially Malignant Oral Lesions These are those pathologies of the oral mucous (oral lichen planus, leukoplakia) that present a tendency for malignant degeneration if some favourable conditions persist (Figure 11). There are conflicting data in literature regarding levels of retinol and beta carotene and the onset of oral lichen planus [31]. Ramaswamy et al. affirmed that folate levels should be investigated in patients with oral lesions

Figure 10: Oral candidosis.

Figure 11: Oral lichen planus.

and symptoms especially those with risk factors of age, poor nutrition, or systemic diseases. When suspected, daily folic acid supplements should be given [32]. With regard to leukoplakia, a significant association has been found with reduced serum levels of vitamins

A, C, and B12, and folic acid (Figure 12). Data in literature confirm that diets rich in fruit and vegetables, above all tomatoes and products derived from them, significantly reduce the risk of the onset of leukoplakia [33]. In a recent report Lodi et al. said that treatment with beta carotene and vitamin A or retinoids was associated with better rates of clinical remission, compared with placebo or absence of treatment. Treatments may be effective in the resolution of lesion; however, relapses and adverse effects are common [34].

#### 12. Micronutrient Deficiencies and Mucosal Disorders

Various types of nutritional deficiencies can produce oral mucosal diseases. Changes such as swelling of the tongue,

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Figure 12: Oral leukoplakia.

papillary atrophy, and surface ulceration are possible in case of micronutrient deficiencies (iron, folate, vitamin B12) [35]. To establish iron, folate, or vitamin B12 deficiency, a hematologic screening that includes complete blood count, red-cell, serum iron, B12, and folate levels should be performed [35, 36]. Although they are rarely required, specific tests for suspected niacin, pyridoxine, and riboflavin deficiency are available [35]. Although glossodynia related to nutritional deficiency is statistically uncommon, it is easily curable with replacement therapy [35]. Identification of a vitamin deficiency through early oral symptoms can forestall development of serious and irreversible systemic and neurologic damage [36]. Deficiencies of vitamin B12 can produce oral signs and symptoms, including glossitis, angular cheilitis, recurrent oral ulcer, oral candidiasis, and diffuse erythematous mucositis. Plummer Vinson syndrome is associated with glossitis and angular cheilitis [35, 36].

#### 37. Which statement is incorrect about Melatonin?

- The pineal gland is the only source of natural melatonin in the human body.
- When melatonin levels increase, your energy level goes down.
- Melatonin protects mitochondria and repairs dysfunctional mitochondria.
- A published human study showed that topical application of melatonin would help heal the inflamed gum tissues of those patients with active periodontal disease.

#### Melatonin levels in periodontal health and disease

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**Background and Objective:** The aim of this study was to measure melatonin levels in the gingival crevicular fluid and saliva of subjects with healthy periodontal tissues, plaque-induced gingival inflammation, chronic periodontitis and aggressive periodontitis.

**Material and Methods:** A total of 70 subjects were examined and assigned to four groups: healthy periodontium (10 subjects); plaque-induced gingival inflammation (20 subjects); chronic periodontitis (20 subjects); and aggressive periodontitis (20 subjects). Gingival crevicular fluid and saliva samples were collected from each subject and analyzed using ELISAs.

Results: The melatonin levels in both gingival crevicular fluid and saliva were lower in patients with chronic periodontitis (10.4 and 12.8 pg/mL, respectively) and aggressive periodontitis (8.4 and 8.8 pg/mL, respectively) than in patients with gingivitis (13.9 and 17.6 pg/mL, respectively) and in healthy subjects (16.6 and 22.9 pg/mL, respectively). The mean melatonin levels in both gingival crevicular fluid and saliva were statistically significantly higher in healthy patients compared with patients with chronic periodontitis and aggressive periodontitis; however, there was no significant difference in the plaque-induced gingival inflammation between the study groups.

Conclusions: The melatonin levels in gingival crevicular fluid and saliva are decreased in diseased periodontal tissues, especially periodontitis. The melatonin level was lowest in the aggressive periodontitis group.

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Periodontal diseases are versatile clinical entities with a complex, multifactorial etiology. The main initiating factor of the most prevalent forms of these diseases is dental plaque, which collaborates with a multitude of local and systemic risk factors, such as smoking, diabetes, putative pathogens and immune defects (1). Plaque-induced gingival inflammation (gingivitis) is the most common form of periodontal disease and may progress to periodontitis, which has several clinical forms. Of these forms, chronic and aggressive periodontitis share the clinical features of bone resorption and clinical attachment loss. The accepted definitions of the two conditions have changed regularly with increased understanding of the disease process (2). Aggressive periodontitis has a rapid rate of disease progression with the absence of large accumulations of dental plaque and calculus, and is seen in otherwise healthy individuals. This form of periodontitis usually affects younger people at or after puberty, and thus can be observed during the second and third decades of life (3). Melatonin is a hormone secreted mainly by the pineal gland and to a lesser extent by the retina, lens, iris, ciliary body, lacrimal gland, skin and gut (4). It is released mainly during night and diffuses passively into saliva via the bloodstream, and can be reliably assayed (5). Although melatonin is present in food such as fruits, vegetables and wheat, the dietary source does not significantly contribute to the circulating levels of melatonin (6).

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The daily variation persists in adulthood with peak serum levels occurring between midnight and 2 AM and is lowest during the day between noon and 2 PM (7). Eventually, in old age, this prominent night-time peak becomes markedly attenuated (8). Melatonin

diffuses passively into saliva via the bloodstream, with the salivary melatonin concentration representing 24–33% of its plasma levels (5). Melatonin exhibits a wide spectrum of activities, including antioxidant functions and protection of the mucosa against various irritants. Furthermore, it protects the oral cavity and the gastrointestinal tract from conditions such as stomatitis, esophagitis, gastritis and peptic ulcer (9). Melatonin appears to be an important modulator of the immune system as it enhances the natural and acquired immunity *in vivo*, and activates monocytes and neutrophils (10). Melatonin has an anti-inflammatory effect (11) and is chemotactic for cultured chick retinal pigment epithelial cells (12). It also stimulates type I collagen synthesis and promotes bone formation (13,14). The relationship between periodontal status and melatonin levels is still unclear and inconclusive (15,16). The present study was conducted to measure melatonin levels in gingival crevicular fluid and saliva from subjects with and without periodontal disease. The null hypothesis assumes that there is no statistically significant difference among the study groups.

#### Material and methods

This study was approved by the Ethics Committee of the Riyadh Colleges of Dentistry and Pharmacy, Riyadh, Saudi Arabia. The exact procedures were explained to all patients who provided signed, informed consent before participation. At the initial examination, detailed medical and dental histories were obtained for each subject and this was followed by a complete periodontal examination. Subjects were excluded if they were smokers, pregnant, lactating, or had uncontrolled systemic disorders or intellectual disability. Patients on medications that could alter melatonin levels (e.g. antidepressants and antipsychotics) were also excluded. Patients who had periodontal treatment in the last 12 mo were not included in the study. Following history-taking and extra-oral and intra-oral examinations, thorough periodontal examinations were performed by the same examiner. Subjects were classified into the following four groups based on the diagnosis of the periodontal condition.

#### Periodontally healthy subjects

Subjects of this group were periodontally healthy; as confirmed by the gingival index of Loe and Silness (17), patients showed no attachment loss and recession with a sulcus depth not exceeding 3 mm. For both saliva and gingival crevicular fluid, sampling time was fixed to around 8.30 PM for all groups.

#### Plaque-induced gingival inflammation

Patients of this group had severe generalized plaque-induced gingival inflammation. Using the gingival index of Loe and Silness (17), patients had severe gingival inflammation, as defined by marked redness, hypertrophy, and tendency for spontaneous bleeding and ulceration. At least 18 teeth were present in each patient (excluding third molars), of which 12 teeth were posterior. Patients in this group had no evidence of attachment loss and had a clear diagnosis of plaque-induced gingival inflammation.

#### Chronic periodontitis

All patients of this group received a thorough periodontal examination. This included assessments of: clinical attachment level; probing depth; hygiene index; gingival index; bleeding on probing; tooth mobility; furcation probing; and gingival recession. Only patients with generalized severe chronic periodontitis were included. Extent was determined based upon the percentage of affected sites according

to Armitage (18). Only patients with a generalized involvement (> 30% of sites involved) were included in this group. The group included patients with at least 18 remaining teeth (excluding third molars) of which at least 12 were molars/premolars. Severity of chronic periodontitis was determined on the basis of the amount of clinical attachment loss ( $\geq 5$  mm) (18). For collection of gingival crevicular fluid samples, four sites were randomly selected to obtain a representative sample for each patient. Owing to the natural random distribution of the disease, samples were not taken from specific areas.

#### Aggressive periodontitis

Patients of this group fulfilled the following criteria.

- 1 Apart from periodontitis, patients were clinically healthy.
- 2 Rapid rate of disease progression, as indicated by the age of the patient and the level of periodontal destruction.
- 3 Inconsistency between the amount of local deposits and the extent of tissue destruction.
- 4 Possible family history.

Full charting and sample collection for patients of this group were performed as previously described for the chronic periodontitis group. Agreement on the clinical diagnosis was made by two clinicians. At the end of data collection, a total of 70 subjects were included in the study. Detailed distribution of the age and gender distribution, and of periodontal clinical parameters, are shown in Table 1.

#### Sample collection

For saliva collection, participants were instructed not to eat, drink, chew gum or brush teeth for at least 30 min before sampling. The mouth was rinsed thoroughly with cold water 5 min before sample collection. To stimulate salivary flow, patients were asked to chew a piece of paraffin wax for 7 min (19). Saliva produced in the first 2 min was discarded, and only

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saliva generated in the remaining 5 min was collected. Samples were centrifuged at 2795 g for 20 min and the supernatant was collected and frozen at  $-20^{\circ}\text{C}$  until required for analysis (20). To obtain gingival crevicular fluid, multiple test sites were randomly selected from the four quadrants. Test sites were dried and isolated with cotton rolls to prevent any contamination with saliva or blood. Before gingival crevicular fluid sampling, supragingival calculus was removed using suitable instrumentation. A standard volume of 5  $\mu\text{L}$  of gingival crevicular fluid was collected extra-crevicularly over a 5 to 10 min time period using a volumetric, microcapillary pipette with a capacity of 1–5  $\mu\text{L}$ . A pooled volume of gingival crevicular fluid was collected from healthy subjects, whereas for subjects with gingivitis and periodontitis, random sampling from four sites was performed. On visual examination, test sites that did not express any gingival crevicular fluid or yielded samples mixed with blood or saliva were excluded. The gingival crevicular fluid samples were stored at  $\leq 20^{\circ}\text{C}$  until evaluated. Collection of samples was performed by the same examiner.

#### Melatonin measurement

The melatonin levels in saliva and gingival crevicular fluid were measured using a competitive immunoassay [DRG® Melatonin Saliva, Marburg, Germany (Non-extraction) (SLV4779); 1]. Direct Saliva Melatonin

ELISA (DRG Instrumentals GmbH, Marburg, Germany) is a competitive immunoassay using a capture antibody technique. The assay follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a nonbiotinylated antigen for a fixed number of antibody-binding sites. The amount of biotinylated antigen bound to the

antibody is inversely proportional to the concentration of analytes of the sample. When the system was in equilibrium, the free biotinylated antigen was removed by washing and the antibody bound to biotinylated antigen was determined using streptavidin peroxidase as the marker and tetramethylbenzidine (TMB) as the substrate. Values of  $> 50 \mu\text{L pg/mL}$  (Standard F) were diluted with Standard A into the linear range of the standard curve (e.g. by dilution 1:10 – example:  $50 \mu\text{L}$  of saliva +  $450 \mu\text{L}$  of Standard A). Dilution was made in glass tubes. The results obtained were multiplied by the dilution factor to obtain corrected results. Values lower than  $0 \text{ pg/mL}$  were not repeated.

#### Statistical analysis

Data were collected and analyzed using Statistical Package for the Social Sciences, version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). First, a two-way analysis of variance (ANOVA) was performed to determine the interaction and the differences among the groups using the two sampling media (saliva and gingival crevicular fluid). ANOVA was followed by Scheffé's test to analyze the means of multiple paired comparisons that was deemed necessary after using ANOVA. An alpha level of 0.05 was used to indicate the statistical significance ( $p \leq 0.05$ ).

#### Results

Melatonin was detected in all samples tested, and the level of melatonin varied among samples and groups. The levels of melatonin in saliva and gingival crevicular fluid showed a progressive decrease from healthy subjects to subjects with aggressive periodontitis. The highest concentration of melatonin was detected in the saliva and gingival crevicular fluid samples of the control group using the two sample sources. The concentration of melatonin in gingival crevicular fluid was  $16.6 \pm 4.2 \text{ pg/mL}$  (mean  $\pm$  standard error) while in saliva, the melatonin concentration was  $22.9 \pm 4.5 \text{ pg/mL}$ . The lowest mean concentration of melatonin was detected in the aggressive periodontitis group ( $8.5 \pm 0.9 \text{ pg/mL}$  in gingival crevicular fluid and  $8.9 \pm 1.0 \text{ pg/mL}$  in saliva). The concentrations of melatonin in both saliva and gingival crevicular fluid of all groups are shown in Table 2 and Fig. 1. Two-way ANOVA was used to determine the effect of media (saliva and gingival crevicular fluid) and subjects (groups) on melatonin levels as a response (dependent) variable. Twoway ANOVA revealed no interaction between media and disease ( $p = 0.764$ ), as indicated in Table 3.

There was no significant difference between the media analyzed ( $p = 0.099$ ), as shown

Table 1. Gender, age and periodontal clinical data

Gender/age distribution Periodontal clinical data

Male Female

Age range (years)

Age (years)

Tooth mobility

Periodontal pocket (mm)

Attachment loss (mm)

Gingival index

Group I (healthy periodontium)

7 3 20 –44  $28.3 \pm 6.7$  0 0 0 0

Group II (gingivitis) 18 2 18–40  $29.9 \pm 7.0$  0 0 0 3 Group III (chronic periodontitis) 16 4 40–

55  $47.2 \pm 4.6$  2  $5.7 \pm 0.5$   $7.2 \pm 0.2$  2 Group IV (aggressive periodontitis) 15 5 28–40  $34.7 \pm$

$5.4$  3  $5.4 \pm 0.5$   $8.0 \pm 0.2$  3

Values are given as mean  $\pm$  SD, except for tooth mobility and gingival index which are given as the mode.

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in Fig. 2. However, upon testing the effect of subject level (groups) on melatonin level, a statistically significant difference was detected ( $p = 0.000$ ), as shown in Table 3. Using Scheffe' s multiple comparisons, significant differences were found upon comparing the healthy periodontium group with the chronic and aggressive periodontitis groups. A statistically significant difference was also found between the gingivitis group and the aggressive periodontitis group. The remaining paired comparisons revealed no statistical difference, as shown in Table 4.

#### Discussion

Periodontal diseases result from the effect of bacterial products, which participate significantly in the disease process, on the host response. It has been suggested that certain biologic mediators contribute significantly to the protection or destruction of periodontal tissues. The well-known destructive factors include the bone-resorbing mediators, specifically, interleukin-1, prostaglandin E2 and tumor necrosis factor alpha (21). Detection and evaluation of local or systemic mediators, of a destructive and/or protective nature, on the periodontium are routinely performed using oral fluids specifically, saliva and gingival crevicular fluid. These fluids are valuable for use in the diagnosis of periodontal diseases as well as for detecting systemic problems. Significant data and evidence have accumulated from the use of gingival crevicular fluid and saliva for the diagnosis of periodontal diseases, as well as from the evaluation of periodontal tissues in health and disease (22,23). In the present investigation, gingival crevicular fluid and saliva were collected to assess melatonin levels and any possible variability of this hormone in periodontal health and disease. Samples were taken during evening (8–9 PM) to ensure that the sampling time would not be affected by the diurnal cycle of melatonin, which peaks between midnight and 2 AM (7). Within the limitations of the current study, although the salivary levels of melatonin were higher than the gingival crevicular fluid levels of melatonin, the difference between both sampling methods was found to be statistically insignificant, confirming the findings of other investigators (15). Therefore, the remaining part of this section will focus on the level of melatonin, regardless of its sampling method. In the current study, it was interesting to find that higher melatonin levels were related to health or

Fig. 1. Melatonin levels in saliva and gingival crevicular fluid samples. Data are presented as mean  $\pm$  standard error.

Table 2. Melatonin levels in saliva and gingival crevicular fluid samples from each study group

Aggressive periodontitis

Chronic periodontitis Gingivitis

Healthy periodontium

Gingival crevicular fluid Saliva

Gingival crevicular fluid Saliva

Gingival crevicular fluid Saliva

Gingival crevicular fluid Saliva

Mean 8.5 8.9 10.4 12.82 13.9 17.6 16.6 22.9 Standard error 0.9 1.0 1.1 1.9 1.8 3.6 4.2 4.5

Sample size (n) 20 20 20 20 20 20 10 10

All values are given as pg/mL.

Table 3. Two-way analysis of variance (ANOVA), performed to test differences in the concentrations of melatonin in saliva and gingival crevicular fluid among the study groups

Source

Type III sum of squares df

Mean square F Significance

Corrected model

33.525 7 4.789 3.617 0.001

Intercept 1569.333 1 1569.333 1.185E3 0.000 Media 3.654 1 3.654 2.760 0.099 Disease

29.255 3 9.752 7.365 0.000 Media 9 disease 1.531 3 0.510 0.385 0.764 Error 174.772 132

1.324 Total 1834.368 140 Corrected total 208.297 139

df, degree of freedom.

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inflammation (gingivitis), but not to periodontal destruction (periodontitis). This was confirmed by the detection of higher levels of melatonin in samples taken from the healthy and gingivitis subjects. On the other hand, lower melatonin levels were found in both aggressive and chronic periodontitis groups compared with healthy and gingivitis groups. Although the melatonin level was lower in the periodontitis groups compared with healthy subjects and the plaque-induced gingival inflammation group, the results revealed no statistically significant difference between the chronic and aggressive forms of periodontitis. This result confirms an earlier report, which revealed that melatonin levels varied from clinically healthy subjects to those with advanced periodontal destruction (15). However,

this is the first study to investigate melatonin levels in aggressive periodontitis. Factors that increase the host susceptibility to periodontal diseases include familial aggregation, single nucleotide polymorphisms, defective neutrophil functions or primed neutrophils, antibodies to bacteria, smoking, stress and herpesvirus infections (24). Therefore, periodontal diseases are multifactorial in etiology. The overall finding of higher levels of melatonin in health may indicate a protective role of this hormone. In addition, lack of melatonin in periodontitis may emphasize the role of remotely produced molecules in the pathogenesis of periodontitis and may add to the overall picture and the currently familiar paradigm of periodontal pathogenesis.

In the study of periodontal disease pathogenesis, some biologic mediators of the host response are protective to periodontal tissues, as in other parts of the body. Antioxidants are protective elements that reduce the quantity of free radicals that are destructive in nature. Many of these free radicals originate from periodontopathic bacteria (25). It has been suggested that an increase in both reactive oxygen and nitrogen during periodontal disease is responsible for the oxidative damage to periodontal tissues (26). The increase in free radical production coexists with a decrease in antioxidant defence. The imbalance between the pro-oxidant and antioxidant systems may lead to further oxidative attack and substantial deterioration of the periodontal tissues (27). Therefore, a factor with an antioxidant effect would counteract the adverse effects of the periodontopathic bacteria, at least indirectly. The exact mechanism through which melatonin may affect the periodontium has not been thoroughly investigated. The well-known effects of melatonin are its antioxidant, antiaging, anti-inflammatory and antimicrobial actions in the medical and dental fields (28). The increased reactive oxygen and nitrogen products that are

present in advanced periodontal disease are responsible for the oxidative damage to the periodontal tissues (27). Melatonin may reduce this oxidative stress and hence participate in tissue protection. It was proposed that the increased generation of free radicals coexists with a decrease in the antioxidant defence mechanisms (29). Therefore, the decrease of melatonin levels in periodontitis might be the result of by-products or mediators that could interfere with the melatonin levels. A decrease of the melatonin level as an antioxidant in advanced periodontal diseases may cause an imbalance between the prooxidant and antioxidant systems, which may lead to a further oxidative attack and a marked deterioration of the periodontal tissues (29). The protective role of melatonin on periodontal tissues might be explained to some extent by its antimicrobial

Table 4. p values of Scheffe' s test for multiple paired comparisons

Healthy periodontium Gingivitis

Chronic periodontitis

Aggressive periodontitis

0.001\* 0.008\* 0.452

Chronic periodontitis

0.050\* 0.326

Gingivitis 0.636 \*Significance at  $p \leq 0.05$ .

Fig. 2. Interaction between the media (saliva and gingival crevicular fluid) and the melatonin level in the different study groups.

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action against *Porphyromonas gingivalis*, *Streptococcus mutans* and *Prevotella intermedia* (15). Melatonin appears to be an important modulator of the immune system as it enhances the natural and acquired immunity in vivo and activates monocytes and neutrophils (10), in addition to its anti-inflammatory effect (11). Melatonin may modulate periodontal destruction by interfering with prostaglandin E2, thereby inhibiting the differentiation of osteoclasts (30). Furthermore, melatonin can modulate some of the proteins that regulate the bone-resorption process in periodontal disease and interact with other biologic agents, such as calcitonin (31). Melatonin may act at the level of the osteoclast lacuna because of its antioxidant properties and its ability to neutralize reactive species, where it inhibits bone resorption (32). It stimulates osteogenic differentiation in bone marrow stem cells, induces the proliferation and differentiation of osteoblasts and increases gene expression of type I collagen, osteopontin, bone sialoprotein and osteocalcin (33,34). The clinical use of melatonin as an immunotherapeutic agent seems promising as the application of melatonin in dental sockets after tooth extraction and placement of endosseous dental implants in beagle dogs resulted in a greater amount of newbone formation in contact with the implant in primary and secondary immunodeficiencies (35). Within the results of the current study, further investigations into the effect of melatonin on immune functions and bone metabolism in the periodontium are recommended. Cutando et al. (7), found that salivary melatonin levels vary according to the degree of periodontal disease. As the degree of periodontal disease increased, the salivary melatonin level decreased, indicating that melatonin may act to protect the body from external bacterial insults. Therefore, melatonin could be potentially valuable in the treatment of periodontal diseases. The present investigation revealed statistically reduced levels of melatonin in subjects with periodontitis as compared with healthy subjects and

those with gingivitis. Reduction of the melatonin level may contribute to depression and insomnia, which are usually associated with aggressive periodontitis. This is the first study to evaluate the levels of melatonin in subjects with aggressive periodontitis. In another study, it was concluded that the systemic manifestations of fatigue, depressive mood, loss of appetite and weight loss were strongly associated with aggressive periodontitis (36). Therefore, the reduced level of melatonin indicates a negative psychologic effect, which may explain the presence of aggressive and severe periodontitis in individuals suffering from stress (37). The overall result of reduction of melatonin and increased stress may contribute to periodontal destruction, which might have an opposite effect to cortisol. Another explanation of the positive effects of melatonin on periodontal structures is its antagonist effects on matrix metalloproteinases, which are key elements of periodontal destruction (38,39). Further investigations are required to elaborate on the possible protective effect of melatonin on specific mediators involved in periodontal destruction. The findings of the current study, although preliminary, may encourage further research on the use of melatonin as an adjunctive diagnostic marker of periodontal disease. Melatonin may be added to the list of gingival crevicular fluid and saliva components currently used to differentiate between periodontitis, which is characterized by attachment loss, and other periodontal conditions without loss of attachment (gingivitis).

#### Conclusions

The following points can be concluded from this study.

1 The levels of melatonin in saliva and gingival crevicular fluid decrease in periodontal disease, especially periodontitis. 2 The decrease in melatonin levels in diseased periodontal tissues might be related to the absence of its protective role or to degradation by inflammatory mediators of periodontal destruction. Further research, particularly on patients taking melatonin as a medication, is recommended.

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Effect of topical application of melatonin on serum levels of C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- $\alpha$ ) in patients with type 1 or type 2 diabetes and periodontal disease

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**Abstract Background:** The present clinical trial study was designed to assess the effect of topical application of melatonin on serum levels of tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-6 (IL-6) and C-reactive protein (CRP) in patients with diabetes and periodontal disease in comparison with healthy controls. **Material and Methods:** Serum levels of TNF- $\alpha$  and IL-6 were measured by enzyme-linked immunosorbent assay and CRP by nephelometry by using the proper commercial kits in 30 patients with diabetes and periodontal disease, and also in a control group of 30 healthy subjects. Periodontograms were performed using the Florida Probe®. Patients with diabetes were treated with a topical application of melatonin (1% orabase cream formula) once daily for 20 days. Healthy subjects were treated with a placebo orabase cream. **Results:** Patients with diabetes and periodontal disease had significantly higher mean levels of serum TNF- $\alpha$ , IL-6 and CRP than healthy subjects ( $P < 0.001$ ). Following topical melatonin application, there was a statistically significant decrease in the gingival index and pocket depth ( $P < 0.001$ ) as well as a significant decrease in IL-6 and CRP serum levels ( $P < 0.001$ ). Local melatonin application in patients with diabetes and periodontal disease resulted in a significant decrease in CRP and IL-6 serum levels as well as an improvement in the gingival index and pocket depth. Patients with periodontal disease had significantly higher serum CRP, IL-6 and TNF- $\alpha$  values by comparison with healthy subjects. **Conclusions:** We conclude that melatonin can modulate the inflammatory action of these molecules in periodontal patients.

**Key words:** Melatonin, periodontal disease, diabetes mellitus, interleukin-6, tumor necrosis factor-alpha, C-reactive protein, inflammatory markers.

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Melatonin and diabetic periodontitis

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**Introduction** Periodontal disease is an inflammatory oral process affecting the alveolar bone, gums, and periodontal ligament (1). Disease status ranges from gingivitis to advanced periodontitis with destruction of connective tissue attachment and alveolar bone which can eventually lead to tooth loss (2). Periodontitis has also been associated with chronic inflammatory conditions including atherosclerosis, cardiovascular disease, rheumatoid arthritis and diabetes mellitus (3-7). Although the precise relationship between systemic conditions and periodontal disease is still unclear, clinical evidence suggests that periodontitis is associated with a systemic host response and a low-level inflammatory state, as assessed by raised serum levels of systemic inflammatory markers (8,9). Severe periodontitis, both in patients with and without diabetes, has been associated with increased serum levels of proinflammatory cytokines and proinflammatory mediators,

including several interleukins (IL), such as IL-7, IL-6, IL-1 $\beta$ , tumor necrosis factor-alpha (TNF- $\alpha$ ), and C-reactive protein (CRP) (4,10-15). Consequently, it has been suggested that periodontal disease may contribute to general inflammation and the development of inflammatory systemic diseases (4,10-14). Furthermore, patients with rheumatoid arthritis receiving anti-TNF- $\alpha$  medication have better periodontal indices and lower levels of TNF- $\alpha$  within the gingival crevicular fluids, which indicates that suppression of proinflammatory cytokines might prove beneficial in suppressing periodontal disease (15). It is known that diabetes is a proinflammatory metabolic disorder that increases the production of cytokines such as IL-6, TNF- $\alpha$  and IL-10 (11-14). Furthermore, it has been suggested that a systemic inflammatory state may be involved in the etiology of this illness (16,17). A relationship appears to exist between periodontal disease and several systemic pathologies-including diabetes mellitus-and it could be hypothesized that controlling one of these two pathologies may be beneficial in controlling the other (17). However, we should remember that periodontal disease is an infectious process with an inflammatory reaction that affects diabetes and glycaemia control. Recent studies have demonstrated that periodontitis itself can cause alterations that the presence of diabetes exacerbates (17). Topical application of melatonin improves these biochemical parameters leading us to consider melatonin is useful in treating this pathology (18). The immunomodulatory effects of melatonin have already been established in patients with and without periodontal disease. A recent systematic review has stated that melatonin may suppress the inflammation of the gingiva and periodontum (19). Melatonin is an indoleamine secreted by the pineal gland following a circadian pattern. As it is highly lipophilic, melatonin reaches every cell in the organism, and also melatonin concentrations in saliva are between 24% and 33% of those found in plasma joined to plasma proteins (20). A significant positive correlation between salivary and plasma melatonin exists and, by measuring salivary melatonin, oral pathologies can be studied in relation to plasma and salivary melatonin behavior. The protective role of the melatonin on the periodontal disease has been reported elsewhere (18,20,21). Furthermore, in a clinical study comparing patients with generalized aggressive periodontitis and periodontally healthy subjects, nonsurgical periodontal therapy for  $\pm$  14 days was associated with a significant decrease in TNF- $\alpha$  and IL-17 serum levels in the group with periodontal disease [10]. To our knowledge, no study to date has evaluated the potential benefits of melatonin on TNF- $\alpha$ , IL-6 and CRP serum levels in subjects with diabetes and periodontitis. Hence, a cross-sectional study was conducted to determine the effect of topical melatonin application on serum TNF- $\alpha$ , IL-6 and CRP concentrations in patients with diabetes and periodontal disease and in a control group of healthy subjects.

**Material and Methods -Participants** The study was conducted at the Healthcare Centre in Pinos Puente (Granada, Spain). A total of 30 healthy individuals of both sexes (20 men, 18 women), aged 31 to 68 years (mean  $\pm$  standard deviation, 47.0  $\pm$  10.3 yrs) without periodontal disease and 30 patients with diabetes and periodontal disease of both sexes (14 men, 16 women) aged 24 to 58 years (mean 43.1  $\pm$  12.4 yrs) were included in the study. There were 17 patients with type 1 diabetes and 13 with type 2 diabetes (Table 1). All patients were free of medication (other than diabetes regimens). Type of periodontal disease was not an inclusion criterion, although most patients presented advanced periodontitis (83.3%) and only 5 patients had mild or moderate periodontal disease. Exclusion criteria included current use of bisphosphonates, oral contraceptives, antibiotic

treatment in the previous 6 months, and having received (within the last 6 months) or currently being treated for diseases of the oral cavity. All healthy subjects were in good general health with no history of systemic disease or clinical signs of periodontal disease. The study was approved by the Ethics Committee of the Faculty of Dentistry of the University of Granada (Spain). Written informed consent was obtained from all participants. -Study Procedures All participants (patients and controls) underwent an oral examination, including medical, dental, and caries assessments. The same dentist performed all examinations. Periodontograms were performed using the Florida Probe® handpiece (computerized periodontal  
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Patients without diabetes (n=30)

Patients with diabetes (n=30) Age (years), mean  $\pm$  SD  $47 \pm 10.3$   $43.1 \pm 12.4$  Women, n (%) 18 (60%) 16 (53%) Men, n (%) 12 (40%) 14 (47%) Type 1 diabetes, n (%) 17 (57%) Type 2 diabetes, n (%) 13 (43%) HbA1c, mean  $\pm$  SD  $8.43 \pm 0.89$  Table 1. Description of the sample (n=60).

probing system). The gingival index and probing depth were recorded in patients with diabetes and in healthy subjects. The diagnosis for periodontal disease was based on probing periodontal pockets  $\geq 4$ mm. The gingival inflammation was evaluated by probing of the gingival margin and observing the spontaneous bleeding of the gingiva. Thereafter, patients with diabetes were treated with a topical melatonin application (1% orabase cream formula) in the upper and lower dental arches on attached gingival surfaces for 20 days. Pure melatonin was purchased from Helsinn Advanced Synthesis, SA (Biasca, Switzerland). Melatonin 1% orabase cream formula was prepared by the Department of Hospital Pharmacy of the Hospital Perpetuo Socorro (Granada, Spain). Quality was certified by Methapharmaceutical Ind. (Barcelona, Spain). Orabase composition: Sodium Carboxymethylcellulose 16.5%; Pectin 16.5%; Jello 16.5%; Plastibase c.s.p.100. Each patient was given the same kind of toothbrush, with the same surface, explaining each patient had to place the pasta with melatonin, without exceeding the limits of the toothbrush. The bore diameter of the tube outlet cream was exactly the same in all samples. The exact dosage, is achieved by means of a simple dispenser, applied to the tube outlet, which supplies a 1cc amount of melatonin vs. placebo preparation. Healthy subjects were treated with a placebo orabase cream. Melatonin cream (or the placebo) was applied daily at night after routine oral hygiene. All participants were instructed how to use the orabase cream. It was recommended they apply the amount that fits on a regular size adult toothbrush to each dental arch. Furthermore, all participants received the same brand of toothpaste for use during the course of the study. Conventional periodontal treatment prior to or during the study was not allowed. Oral examinations were also performed at the end of treatment (20 days after). -Measurements of TNF- $\alpha$ , IL-6 and CRP Serum Concentrations In all participants (patients with diabetes and periodontal disease and healthy controls), blood samples were collected in fasting conditions at baseline and after treatment with melatonin or the placebo. Serum was separated from blood by centrifuging (10 min at 1300 rpm), stored in aliquots at  $-80^{\circ}\text{C}$ , and thawed immediately before assay. Aliquots of each sample were assayed using commercially available TNF- $\alpha$  and IL-6 sensitive ELISA (R & D Systems Inc.,

Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The lower limits of detection were 0.016 pg/mL for IL-6 and 0.06 pg/mL for TNF- $\alpha$ . The CRP concentration in serum was measured using nephelometry, a commercially available latex particle-enhanced immunoassay (NA Latex CRP kit, Dade Behring, Tokyo, Japan). -Plasma melatonin determination Patients came to the laboratory at 08:00 after an overnight fast and remained seated for 30 min before samples were taken. Blood samples were drawn from the antecubital vein and centrifuged at 3000 g for 10 min. Plasma was separated and frozen at -20°C until analysis. Plasma melatonin was determined by a commercial RIA (DVD Biochemie, Marburg GmbH, Germany) and quality control was performed showing intra- and interassay coefficients of variation of 11.3% and 6.3%, respectively. The recovery of added melatonin was 84.4% and assay sensitivity was 4.65 pg/mL. -Statistical Analysis Quantitative variables are expressed as mean  $\pm$  standard deviation (SD). The paired Student's t test was used to compare the gingival index and probing depth before and after topical application of melatonin in patients with diabetes and periodontal diabetic disease, and the Student's t test for independent samples to compare TNF- $\alpha$ , IL-6 and CRP serum levels between patients with diabetes and healthy subjects. The relationship between the gingival index and probing depth with TNF- $\alpha$ , IL-6 and CRP serum levels was assessed with Pearson's correlation coefficient. Statistical significance was set at  $P < 0.05$ . SPSS 11.0 was used to analyze data. Results Comparison of TNF- $\alpha$ , IL-6 and CRP serum levels in patients with diabetes + periodontal disease and healthy

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controls before topical treatment with melatonin is shown in table 2. Patients with diabetes and periodontal disease had significantly higher mean levels of serum TNF- $\alpha$  ( $1.79 \pm 0.19$  vs  $0.82 \pm 0.17$  pg/mL), IL-6 ( $0.57 \pm 0.07$  vs  $0.38 \pm 0.05$  pg/mL), and CRP ( $0.39 \pm 0.11$  vs  $0.21 \pm 0.08$  mg/L) by comparison with healthy subjects. Moreover patients with diabetes had significantly lower values of salivary melatonin ( $4.5 \pm 0.81$  vs  $2.7 \pm 0.81$  pg/ mL) and plasma melatonin ( $13.9 \pm 3.87$  vs  $9.7 \pm 3.27$  pg/ mL) than healthy subjects. In periodontal terms, after a daily topical melatonin application during 20 days among diabetic+periodontal patients, there was a statistically significant decrease in the gingival index ( $15.84 \pm 10.26$  vs  $5.59 \pm 4.08$ ) and pocket depth ( $28.29 \pm 19.48$  vs  $11.90 \pm 9.01$ ). Moreover it was observed a significant decrease in serum levels of IL-6 ( $0.57 \pm 0.07$  vs  $0.47 \pm 0.07$  pg/mL) and CRP ( $0.39 \pm 0.11$  vs  $0.31 \pm 0.11$  mg/L). However, no significant differences were found in serum TNF- $\alpha$  levels before and after treatment with topical melatonin (Table 3). Furthermore, it was found that TNF- $\alpha$ , IL-6 and CRP of serum levels before and after topical melatonin treatment were significantly correlated with the gingival index and pocket depth before and after treatment (Table 4). This means that a decrease in the gingival index and pocket depth was linearly proportional with the decrease in TNF- $\alpha$ , IL-6 and CRP serum levels after topical melatonin treatment.

Discussion The present results confirm the beneficial effect of topical melatonin application on clinical parameters of periodontal disease, such as the gingival index and pocket depth. Moreover, it was confirmed that the severity of the periodontal disease and the serum levels TNF- $\alpha$ , IL-6 and CRP were significantly correlated. Before melatonin treatment, serum TNF- $\alpha$ , IL-6 and CRP levels in diabetic patients with periodontal disease were significantly higher than in healthy control subjects. It should be taken into account that

human adipose tissue is a potent source of inflammatory interleukins that could cause a false relationship between periodontal illness and the presence of interleukins (22). After topical melatonin treatment, there was a statistically significant decrease in the gingival index and pocket depth, as well as a significant decrease in serum levels of IL-6 and CRP. This, together with the fact that the melatonin was used in topical form, supports our belief that the origin of the interleukins is, basically, a consequence of periodontal disease. The results obtained by using topical melatonin appear to be better than those obtained by other authors using nonsurgical treatment of periodontal disease (23). However, not all authors agree that treating periodontal disease reduces interleukin concentrations in serum (24). The present results demonstrate that diabetic patients with untreated periodontitis present increased circulating levels of markers of inflammation and proinflammatory

Healthy subjects (n = 30) mean ± SD

Diabetic Patients (n = 30) mean ± SD

P value

TNF- $\alpha$ , pg/mL 0.82 ± 0.17 1.79 ± 0.19 < 0.001 IL-6, pg/mL 0.38 ± 0.05 0.57 ± 0.07 < 0.001

CRP, mg/L 0.21 ± 0.08 0.39 ± 0.11 < 0.001

Table 2. Comparison of serum TNF- $\alpha$ , IL-6 and CRP levels in diabetic patients with periodontal disease and healthy controls before topical treatment with melatonin.

Table 3. Comparison of gingival index, pocket depth and TNF- $\alpha$ , IL-6 and CRP serum levels before and after topical application of melatonin in patients with diabetes and periodontal disease.

Treatment with topical melatonin for 20 days P-value Before (mean ± SD) After (mean ± SD) Periodontal indexes Gingival index 15.84 ± 10.26 5.59 ± 4.08 < 0.001 Pocket depth 28.29 ± 19.48 11.90 ± 9.01 < 0.001 Serum levels TNF- $\alpha$ , pg/mL 1.79 ± 0.19 1.76 ± 0.19 NS IL-6, pg/mL 0.57 ± 0.07 0.47 ± 0.07 < 0.001 CRP, mg/L 0.39 ± 0.11 0.31 ± 0.11 < 0.001

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Table 4. Correlations between gingival index, pocket depth and TNF- $\alpha$ , IL-6 and CRP levels in serum before and after treatment with melatonin in the patient group (n=30). Gingival index Pocket depth Before After Before After TNF- $\alpha$ , pg/mL Before r = 0.610, P < 0.001 r = 0.526, P = 0.003 r = 0.658, P < 0.001 r = 0.459, P = 0.01 After r = 0.620, P < 0.001 r = 0.514, P = 0.004 r = 0.673, P < 0.001 r = 0.470, P = 0.009 IL-6, pg/mL Before r = 0.569, P = 0.01 r = 0.484, P = 0.007 r = 0.609, P < 0.001 r = 0.417, P = 0.022 After r = 0.625, P < 0.001 r = 0.523, P = 0.003 r = 0.663, P < 0.001 r = 0.461, P = 0.01 CRP, mg/L Before r = 0.635, P < 0.001 r = 0.593, P = 0.001 r = 0.693, P < 0.001 r = 0.527, P = 0.003 After r = 0.687, P < 0.001 r = 0.565, P = 0.001 r = 0.730, P < 0.001 r = 0.573, P = 0.001 Pocket depth Before r = 0.943, P < 0.001 r = 0.721, P = 0.005 After r = 0.701, P = 0.07 r = 0.684, P = 0.009 cytokines (CRP, TNF- $\alpha$  and IL-6). Over expression of these inflammatory mediators in serum in subjects with periodontal disease suggests a potential hyper-reactivity of cells in these individuals that may favour periodontal tissue breakdown and a systemic inflammatory burden. Elevated levels of cell-mediated and cytokine-mediated markers of inflammation-including CRP and other cytokines-are reported to be associated with periodontal disease (10). In systematic reviews and meta-analyses of CRP protein in relation to periodontitis, CRP levels were consistently higher in patients than in controls

(25,26). It has been estimated that the weighted mean difference in CRP between patients and controls was 1.56 mg/L ( $p < 0.00001$ ) and that the levels of CRP after periodontal therapy, is reduced on average 0.50 mg/L (95% confidence interval [CI] 0.08-0.93) (25). Moreover, a metaanalysis of 4 randomized clinical trials (26) estimated a significant reduction in CRP levels (-0.231 mg/L on average) after introducing periodontal treatment, which indicates that nonsurgical periodontal treatment had a positive effect on reduction of serum CRP levels. The present results agree with findings from both metaanalyses, although in our study periodontal treatment was replaced by topical application of melatonin, the beneficial effect of which on periodontal disease has been demonstrated in previous studies (20,21,27). The systemic levels of TNF- $\alpha$  in subjects with periodontitis and the impact of periodontal treatment on serum TNF- $\alpha$  concentrations are little understood. We found significantly higher TNF- $\alpha$  levels among patients with diabetes and periodontal disease than in healthy subjects however, the TNF- $\alpha$  levels before and after melatonin use did not change significantly, therefore this serum parameter seems to be indifferent to the periodontal chan

ges, in contrast to that reported elsewhere (28). Higher levels of circulating TNF- $\alpha$  in patients with periodontitis as compared with healthy subjects have consistently been reported in most studies (10,24). However, the effect of periodontal therapy on the systemic level of TNF- $\alpha$  has not been clarified. Mendes Duarte et al. (10) report that subjects with generalized aggressive periodontitis presented higher levels of circulating TNF- $\alpha$  than controls but TNF- $\alpha$  levels remained higher after periodontal therapy. Nakajima et al. (29) report that periodontal treatment did not affect TNF- $\alpha$  levels We are aware that Type I diabetes, is more aggressive and could be a determining factor to the effect of melatonin, but the sample size is insufficient to find significant differences between these subgroups. As reported elsewhere we found high levels of IL-6 in patients with periodontal disease when compared with healthy controls. The IL-6 is an important proinflammatory cytokine involved in the regulation of host response to tissue injury and infection. It is produced by a variety of cells, such as monocytes, fibroblasts, osteoblasts and vascular endothelial cells, in response to inflammatory challenges (27). Subjects included in the study were not instructed to change their habits of toothbrushing, therefore, we believe that the results are attributable to the effect of melatonin, but it is possible that, some subjects, change their oral hygiene habits when they entered the study; in fact, some studies show a reduction of IL-1 $\beta$ , only with changing brushing techniques (30). Future studies, should include positive and negative controls and tests, ie healthy patients with periodontitis and diabetic patients without periodontitis, to avoid confusion of diabetes factor in the outcome (periodontitis).

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To summarize, the diabetic patients with periodontal disease had significantly higher serum CRP, IL-6 and TNF- $\alpha$  values by comparison with healthy subjects. After a 20 days of daily local melatonin application in such diabetic patients with periodontal disease, a significant decrease in serum CRP and IL-6 levels as well as an improvement in the gingival index and pocket depth was observed.

## Melatonin Receptor Agonists as the “Perioceutics” Agents for Periodontal Disease through Modulation of Porphyromonas gingivalis Virulence and Inflammatory Response

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Abstract

Aim

“Perioceutics” including antimicrobial therapy and host modulatory therapy has emerged as a vital adjunctive treatment of periodontal disease. Melatonin level was significantly reduced in patients with periodontal diseases suggesting melatonin could be applied as a potential “perioceutics” treatment of periodontal diseases. This study aims to investigate the effects of melatonin receptor agonists (melatonin and ramelteon) on Porphyromonas gingivalis virulence and Porphyromonas gingivalis-derived lipopolysaccharide (Pg-LPS)-induced inflammation.

Methods

Effects of melatonin receptor agonists on Porphyromonas gingivalis planktonic cultures were determined by microplate dilution assays. Formation, reduction, and viability of Porphyromonas gingivalis biofilms were detected by crystal violet staining and MTT assays, respectively. Meanwhile, biofilms formation was also observed by confocal laser scanning microscopy (CLSM). The effects on gingipains and hemolytic activities of Porphyromonas gingivalis were evaluated using chromogenic peptides and sheep erythrocytes. The mRNA expression of virulence and iron/heme utilization was assessed using RT-PCR. In addition, cell viability of melatonin receptor agonists on human gingival fibroblasts (HGFs) was evaluated by MTT assays. After pretreatment of melatonin receptor agonists, HGFs were stimulated with Pg-LPS and then release of cytokines (IL-6 and IL-8) was measured by enzymelinked immunosorbent assay (ELISA).

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## Results

Melatonin and ramelteon did exhibit antimicrobial effects against planktonic culture. Importantly, they inhibited biofilm formation, reduced the established biofilms, and decreased biofilm viability of *Porphyromonas gingivalis*. Furthermore, they at sub-minimum inhibitory concentration (sub-MIC) concentrations markedly inhibited the proteinase activities of gingipains and hemolysis in a dose-dependent manner. They at sub-MIC concentrations significantly inhibited the mRNA expression of virulence factors (*kgp*, *rgpA*, *rgpB*, *hagA*, and *ragA*), while increasing the mRNA expression of ferritin (*ftn*) or hemolysin (*hem*). They did not show obvious cytotoxicity toward HGFs. They inhibited Pg-LPS-induced IL-6 and IL-8 secretion, which was reversed by luzindole, the melatonin receptor antagonist.

## Conclusion

Melatonin receptor agonists can inhibit planktonic and biofilm growth of *Porphyromonas gingivalis* by affecting the virulent properties, as well as Pg-LPS-induced inflammatory response. Our study provides new evidence that melatonin receptor agonists might be useful as novel “perioceutics” agents to prevent and treat *Porphyromonas gingivalis*-associated periodontal diseases.

**Introduction** Periodontal diseases have a global distribution and all types of periodontal disease are infectious disorders resulting from the interplay between pathogenic agents and host immune reactions [1]. “Perioceutics” is addressed the use of pharmacotherapeutic agents including antimicrobial therapy and host modulatory therapy, specifically developed to manage periodontal disease [2–4]. “Perioceutics” has emerged as a vital adjunctive treatment of periodontal disease [3,4]. Hence, the development of the “perioceutics” agents is of great significance in the better management of periodontal disease. Melatonin (N-acetyl-5-methoxytryptamine) is a well-studied, endogenous molecule that regulates and modulates a wide variety of physiological functions. Besides its important role as a chronobiotic, melatonin has been found to be involved in a variety of pathophysiological processes including the modulation of immune response, anti-inflammatory, antioxidant, antitumoral, neuroprotective processes [5–7]. Recent studies have shown that melatonin level was significantly reduced in patients with periodontal diseases, suggesting melatonin be applied as an important biomarker in the diagnosis and also potential treatment of periodontal diseases [7–11]. A cross-sectional study has demonstrated that topical application of melatonin on gingival tissues has a favorable effect on preventing the periodontal disease [12]. In vivo studies were successfully performed with melatonin in periodontal disease models indicating that melatonin could exert protective and preventive effects [13–15]. Choi et al. reported that melatonin inhibited

*Prevotella intermedia* LPS-induced inflammation [16]. As is well known, microbial plaque has been recognized as the primary etiology for the development of periodontal disease [17,18]. Among the oral microbiota in periodontal disease, *Porphyromonas gingivalis*, as a Gram-negative, rod-shaped, and anaerobic bacterium, is strongly implicated as a “keystone” periodontal pathogen [1,19,20]. *Porphyromonas gingivalis* utilizes multiple virulence factors, such as proteinases (e.g. gingipains), fimbriae, LPS, and Melatonin and Ramelteon Inhibit *P. gingivalis* Growth and Inflammation PLOS ONE | DOI:10.1371/journal.pone.0166442 November 10, 2016 2 / 20 University and Research Institute by Shanghai Education Commission. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist. cytotoxic and hemolytic molecules [21–24]. Several genes involved in virulence of *Porphyromonas gingivalis* have been identified, for example, *kgp*, *rgpB*, *vimA*, *ragA*, and hemagglutinin (*hagA*), et al [24]. Activity of these virulence factors is critical and essential for the growth and survival of *Porphyromonas gingivalis* as nutrients. Virulence factors are helpful to adhere to lots of host cell surfaces and play vital roles in the breakdown of host-defense mechanisms, the penetration and progressive damage of the host connective tissues [25,26]. Furthermore, virulence factors such as LPS, can stimulate the production of inflammatory factors, for example interleukins (IL) [26], which are involved in the activation and development of inflammation in periodontal pockets. To date, only a few involve the in vitro antimicrobial activities of melatonin in infectious diseases, and its effect remains controversial. For example, Elmahallawy et al. and Schuck et al. reported that melatonin exhibited anti-parasite activity [27,28]. Tekbas reported that melatonin exerted a potent antimicrobial activity on multidrug-resistant, gram-positive and gram-negative bacteria [29]. Ozturk et al. reported that 300 µg/mL melatonin inhibited *Candida albicans* [30]. Conversely, Wang et al. and Konar et al. performed in vitro antimicrobial studies of melatonin and demonstrated that antimicrobial properties of it are limited [31,32]. Gomez Florit reported that melatonin did not affect *Staphylococcus epidermidis* growth [8]. However, there is no report on the antimicrobial activity of melatonin against oral pathogens. Ramelteon, a melatonin derivative, was the first melatonin receptor agonist approved for human use. In 2005, the US Food and Drug Administration (FDA) licensed it in the USA for the treatment of insomnia [33]. Compared to melatonin, ramelteon has a higher lipophilicity and it is more easily taken up and retained by tissues, so ramelteon can be of much therapeutic value. Therefore, the present study aims at evaluating the “periocutaneous” therapeutic potential of melatonin receptor agonists (melatonin and ramelteon) on antimicrobial activity and anti-inflammatory action against *Porphyromonas gingivalis*, a “keystone” periodontal pathogen.

**Materials and Methods**  
**2.1 Chemicals, bacteria and culture conditions**  
 Commercial melatonin was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Ramelteon was purchased from Selleck Chemicals (Houston, TX, USA) and luzindole was purchased from Tocris Bioscience (Bristol, UK). Test samples were prepared by dissolving melatonin and ramelteon in dimethyl sulfoxide (DMSO), and the indicated concentration was reached through dilution with Tryptic Soy Broth (TSB; Bacto™, BD, Sparks, MD, USA) or Dulbecco’s Modified Eagles Medium (DMEM, Hyclone, Logan, UT, USA). DMSO was used as the vehicle control in the experiments. The test bacterium *Porphyromonas gingivalis* ATCC33277 was

provided by the Shanghai Research Institute of Stomatology and Shanghai Key Laboratory of Stomatology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China) and grown in fresh TSB at 37°C under anaerobic conditions (80% N<sub>2</sub>, 10% H<sub>2</sub>, 10% CO<sub>2</sub>).

**2.2 Bacteria antibiotic susceptibility assay** The drug susceptibility of planktonic cultures was determined by a 2-fold serial dilution method [29]. Dilutions of melatonin (3.13–1600 µg/mL) and ramelteon (3.13–800 µg/mL) were prepared in TSB, and added in a flat-bottomed 96-well microplate. A 20 µL quantity of *Porphyromonas gingivalis* suspension at a final concentration of 1×10<sup>7</sup> colony-forming units (CFU)/mL was added and incubated under anaerobic conditions for 48 h at 37°C. Control wells that were not inoculated or that contained the vehicle control were also prepared. Bacterial growth was monitored at the wavelength of 630 nm. The MIC referred to the lowest

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concentration of melatonin at which inhibited growth to a level of < 0.05 at 625 nm, i.e. no macroscopically visible growth [29]. To determine the minimal bactericidal concentration (MBC) values, an aliquot of 10 µL cell suspension from each well was taken, and bacterial clones were counted after incubation for 3–5 days at 37°C under anaerobic conditions. MBC referred to the lowest concentration at which any colony did not grow on the agar.

**2.3 Biofilm susceptibility assay** The effects on *Porphyromonas gingivalis* biofilm formation were examined by the crystal violet staining method [34,35]. A 200 µL aliquot of *Porphyromonas gingivalis* suspension (1×10<sup>7</sup> CFU/mL) was grown in TSB supplemented with drugs for 48 h at 37°C in a 96-well microplate. The culture supernatant was removed and the adherent biofilms were rinsed for three times using phosphate buffered saline (PBS). Then, the adherent biofilms were incubated with methanol for 15 min followed by staining with 0.04% (w/v) crystal violet for 15 min. After washing with deionized water, the microplate was dried overnight. Finally, 95% ethanol was used to dissolve and the microplates were shaken for 1 h at room temperature. The OD values were recorded at wavelength of 550 nm. Definition of the minimum biofilm inhibition concentration (MBIC) was the lowest drug concentration at which at least 50% of biofilms was inhibited compared with the control (MBIC<sub>50</sub>) [34]. The effects of melatonin and ramelteon on mature *Porphyromonas gingivalis* biofilm reduction were examined by the crystal violet staining method [34,35]. A 200 µL *Porphyromonas gingivalis* suspension (1×10<sup>7</sup> CFU/mL) was added to the 96-well microplate so as to develop the biofilms. The culture medium was decanted carefully with the integrity of biofilms under anaerobic incubation for 48 h at 37°C. The formed biofilms were gently rinsed with PBS and non-adherent cells were removed. Bacterial cells were incubated in the presence of drugs for 24 h. Then, the media were removed and biofilms were gently washed with PBS and subsequently fixed with methanol. The biofilms were stained with 0.04% crystal violet and recorded using a microplate reader. The minimum biofilm reduction concentration (MBRC) was the lowest drug concentration at which at least a 50% (MBRC<sub>50</sub>) of the biofilms were reduced compared with the control [34]. The effect of melatonin and ramelteon on the viability of in vitro *Porphyromonas gingivalis* biofilms were determined using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) method modified from that of Tang HJ et al [36]. The *Porphyromonas gingivalis* biofilm formation, incubation of the biofilm and the drugs were the same as described for the above method. MTT (0.5 mg/mL)

was added to detect the metabolic activity of the biofilms. All plates were cultured in the dark for 2 h at 37°C. Following incubation, MTT solution was gently aspirated from each well, and 100µL of DMSO was added to dissolve the formazan crystals. After the plate was shaken for 10 min at room temperature in the dark, absorbance at 490 nm was recorded using a microplate reader. The sessile MIC (SMIC50) was the lowest drug concentration at which there was at least a 50% reduction compared with that of the control [34].

**2.4 Biofilm measurement by CLSM** *Porphyromonas gingivalis* biofilms, grown as described above assay of biofilm formation for 48 h on glass coverslips, were formed in the presence or absence of sub-MIC drugs. Bacterial cells were stained with the LIVE/DEAD1 BacLight™ Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR, USA). After staining for 15 min out of the light, CLSM (Leica TCS SP2; Leica DMIRE2 Microsystems, Wetzlar, Germany) was applied to acquire images and thickness

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of biofilm was measured. Live bacteria showed fluorescent green, whereas dead bacteria were fluorescent red. The exciting laser intensity, background level, contrast and electronic zoom were fixed for each experiment. In each experiment, at least four random fields were recorded.

**2.5 Gingipain activity assays** The effect of melatonin and ramelteon on gingipain activity of *Porphyromonas gingivalis* was measured according to the previous methods [34,37].

Briefly, a 24 h *Porphyromonas gingivalis* culture ( $1 \times 10^8$  CFU/mL) in the presence of sub-MIC levels of drugs was centrifuged ( $10,000 \times g$  for 15 min at 4°C), washed and suspended in PBS at an OD<sub>630</sub> of 2 for *Porphyromonas gingivalis* Arg-gingipains (Rgp) activity and OD<sub>630</sub> of 1 for *Porphyromonas gingivalis* Lysgingipain (Kgp) activity. The *Porphyromonas gingivalis* cells were incubated with PBS with or without drugs at sub-MIC concentrations of drugs, a specific substrate for Rgp (0.4 mM, benzoyl-arginine-p-nitroanilide, BAPNA) or Kgp (0.4 mM, N-p-tosyl-glycine-proline-lysine-p-nitroanilide, TGPpNA), and a reductant (1 mM, DL-Dithiothreitol, DTT). The mixtures were incubated at 37°C in the dark. Activity of Rgp and Kgp indicated as the hydrolysis of the Rgp and Kgp-specific chromogenic substrates was detected at wavelength of 405 nm. Substrates with drugs and substrates alone were individually used as controls. A 100% value was defined as the degradation after a 4 h-treatment without drugs.

**2.6 Hemolytic activity assay** Hemolytic activity was performed as previously reported [38,39]. In brief, *Porphyromonas gingivalis* cells were centrifuged ( $10,000 \times g$  for 10 min), washed three times with PBS, and then re-suspended to a final OD<sub>600</sub> of 1.5. At the same time, sheep erythrocytes were harvested by centrifugation ( $4,400 \times g$  for 20 min) and washed with PBS until the supernatant did not contain hemoglobin pigment visibly. The sheep erythrocytes at a concentration of 1% were suspended in PBS and then mixed with an equal volume of bacterial cells with or without drugs at sub-MIC concentrations of drugs at 37°C for 4 h. Then samples were further spun at  $1,300 \times g$  for 5 min and the supernatant was detected. The hemolytic activity was determined at wavelength of 405 nm.

Erythrocytes were used alone as a negative control. Complete erythrocytes lysis was obtained by mixing 10µL of a 10% (w/v) sodium dodecyl sulfate (SDS) solution with 1% (v/v) sheep erythrocytes. Relative hemolytic activity was determined compared to the vehicle group.

**2.7 Quantitative analysis of gene expression by RT-PCR** The total RNA of *Porphyromonas gingivalis* was extracted by a Bacterial RNA Kit (Omega Bio-Tek, Norcross, GA, USA), and measured by Nanodrop2000 to determine the RNA concentration. Reverse transcription was performed by M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) to generate cDNA. Amounts of mRNA transcripts were measured by the Roche LightCycler 480 real-time PCR detection system (Roche, Basel, Switzerland). The galE gene was used as the housekeeping amplicon [40]. Reactions were performed with 20 µL of a mixture containing 10 µL of SYBR Premix Ex Taq II (2×; Takara), 1.6 µL of each gene-specific primer, 3.4 µL of sterile distilled water and 5 µL of the cDNA template. The forward and reverse primer sequences are shown in Table 1 [40,41]. Real-time PCR conditions included 30 s at 95°C; 10 s at 95°C, 20 s at 60°C and 15 s at 72°C for 40 cycles. Melting temperature curve analyses were used to validate the specificity of each primer pair. Data were analyzed using the comparative Ct method.

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**2.8 Ethics statement, cell culture and cytotoxicity assay** This study was approved by the Ethics Committee of the Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine in May, 2015 (Approval Number: 2015– 41). The forms of informed consents were obtained from the participants prior to taking part in this research. Primary cultures of HGFs were excised from human gingival tissue. Gingival tissue explants were obtained from patients undergoing crown-lengthening surgery at the Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine in June, 2015. Gingival specimens were washed in 70% ethanol, and then in 100 units/mL penicillin G and 100 µg/mL streptomycin. They were cut into small pieces and maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 15% heat-inactivated fetal bovine serum, 100 U/mL of penicillin G and 100 µg/mL of streptomycin at 37°C in 5% CO<sub>2</sub> and humidified air. The cells were used for the experiments between passage 2 and passage 10. The cell culture medium of HGFs was then replaced by serial dilutions of melatonin and ramelteon, with final concentrations ranging from 1 µg/mL to 400 µg/mL. DMEM without drugs served as a negative control. After 24 h of culture, MTT dye (0.5 mg/mL) was added and then incubated for 4 hours at 37°C. Following incubation, MTT was aspirated from each well, and 100 µL of DMSO was added to dissolve the formazan crystals. The plate was then shaken for 10 min at room temperature. The optical density (OD) values for each well were measured at 490 nm using a microplate reader (ELx800, Biotek Instruments, Winooski, VT, USA). A higher absorbance means a higher formazan concentration, indicating higher metabolic viability of HGFs in the wells.

Table 1. The primers sequence of genes used in RT-PCR.

Target gene	Description	Primer sequence
rgpA	arginine-specific cysteine proteinase	RgpA Forward:5'-CACCGAAGTTCAAACCCCTA-3' Reverse:5'-GAGGGTGCAATCAGGACATT-3'
rgpB	arginine-specific cysteine proteinase	RgpB Forward:5'-GCTCGGTCAGGCTCTTTGTA-3' Reverse:5'-GGGTAAGCAGATTGGCGATT-3'
kgp	lysine-specific cysteine proteinase	Kgp Forward:5'-AGGAACGACAAACGCCTCTA-3' Reverse:5'-GTCACCAACCAAAGCCAAGA-3'
hagA	hemagglutinin protein	HagA Forward:5'-TAAATAAGGGCGGAGCAAGA-3' Reverse:5'-GACGGAAAGCAACATACTTCG-3'
ragA	receptor antigen A	ragA Forward:5'-CGCTATTCTTCTTTGCTTGCT-3' Reverse:5'-GATCGTGGTGTTCGACAA-3'
vimA	virulence modulating gene A	vimA Forward:5'-TCGCGTAGTCTGAGAGTAACCTT-3' Reverse:5'-

GGTATAAACGAAGACAGCAGCAG-3' ftn ferritin Forward:5'-CGGCGAGGTGAAGATAGAAG-3'  
Reverse:5'-CTCCTGAGAGAGACGGATCG-3' hem hemolysin Forward:5'-  
ACGAAGCCTTGTCTCCTCA-3' Reverse:5'-CAATGAATATGCCGGTTTCC-3' hmuR TonB-  
dependent receptor HmuR Forward:5'-CTCCCATGCGGCCAACCTCC-3' Reverse:5'-  
GCAGACGGGCTGTACGGCTACC-3' luxS S-ribosylhomocysteine lyase Forward:5'-  
GAATGAAAGAGCCCAATCG-3' Reverse:5'-GTAATCGCCTCGCATCAG-3' gale UDP-glucose 4-  
epimerase Forward:5'-TCGGCGATGACTACGACAC-3' Reverse:5'-  
CGCTCGCTTTCTCTTCATTC-3' doi:10.1371/journal.pone.0166442.t001

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2.9 Measurement of IL-6 and IL-8 HGF cells were treated with melatonin receptor agonists for 6 h, followed by Pg-LPS (InvivoGen, San Diego, USA) at 1µg/mL concentration for 24 h. The release of IL-6 and IL-8 into the supernatant was measured using the commercial ELISA kits (Xi'tang biotechnology, shanghai, china), according to the manufacturer's instructions.

2.10 Statistical analysis Data were presented as the means±SEM. Statistical analysis was performed with the Student t-test and a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparison test. The difference was considered statistically significant at  $P < 0.05$ .

Results 3.1 The antimicrobial activity of melatonin and ramelteon against *Porphyromonas gingivalis* planktonic culture Results of the susceptibility assay of *Porphyromonas gingivalis* planktonic cultures to melatonin and ramelteon are summarized in Table 2. Both drugs showed a dose-dependent inhibition on in vitro growth of *Porphyromonas gingivalis* (Fig 1). As shown in Table 2 and Fig 1, the MIC and MBC of Melatonin were 100µg/mL and 1600µg/mL, respectively, while those of ramelteon were 50µg/mL and 400µg/mL, respectively.

3.2 Effect of melatonin and ramelteon on *Porphyromonas gingivalis* biofilms In addition to growth inhibition of *Porphyromonas gingivalis* planktonic culture, melatonin and ramelteon also noticeably showed antimicrobial activity against *Porphyromonas gingivalis* biofilms. Biofilm formation by *Porphyromonas gingivalis* considerably decreased in response to melatonin and ramelteon in a dose-dependent manner (Fig 2). As shown in Table 2, the MBIC50 values of melatonin and ramelteon were 50µg/mL and 25µg/mL, respectively. Furthermore, the mature biofilm attached at the bottom of the microplate for 48 h was reduced by melatonin (MBRC50 = 200µg/mL) and ramelteon (MBRC50 = 100µg/mL). In addition, melatonin and ramelteon decreased biofilm metabolic activity, suggesting that the bacterial cells' viability within the biofilm was inhibited by the drugs (SMIC50 = 100µg/mL for melatonin; SMIC50 = 50µg/mL for ramelteon). To further determine the effect of melatonin and ramelteon at sub-MIC concentrations on biofilm formation, 2-day-old biofilms of *Porphyromonas gingivalis* were observed by CLSM. The biofilms were made up of viable *Porphyromonas gingivalis* (Fig 3). When treatment of 50µg/mL melatonin or 25µg/mL ramelteon, there were much more dead cells observed in Table 2. Antimicrobial effect of melatonin and ramelteon against *P. gingivalis* planktonic and biofilm.

Drug	Planktonic <i>P.gingivalis</i>	<i>P.gingivalis</i> biofilm	MIC(µg/mL)	MBC(µg/mL)	Formation Reduction Viability	MBIC50 (µg/mL)	MBRC50 (µg/mL)	SMIC50 (µg/mL)
Melatonin	100	1600	50	200	100	50	200	100
Ramelteon	50	400	25	100	50			

More than three individual experiments were used for the measurement.

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biofilms compared to the control group. The volumes of *Porphyromonas gingivalis* biofilms with 50 µg/mL melatonin or 25 µg/mL ramelteon were smaller and thinner than that of the control (Fig 3A). In the control biofilms, *Porphyromonas gingivalis* cells aggregated clusters. Conversely, bacterial cells were interspersed in melatonin- and ramelteon-treated biofilms. In addition, the average thicknesses of biofilms were determined by CLSM (Fig 3B). The thicknesses of melatonin-treated biofilms and ramelteon-treated biofilms were 26.7±2.2 µm and 30.1±1.1 µm, respectively, which were thinner than the control group (38.2±1.5 µm). These data demonstrated that melatonin and ramelteon inhibited biofilm formation, reduced the established biofilms, and decreased biofilm viability.

Fig 1. The number of colonies after 48 h-incubation of *Porphyromonas gingivalis* in blood agar. (A) The top six areas represent melatonin-containing wells; the bottom six areas are vehicle (DMSO) only-containing wells. (B) The top six areas represent ramelteon-containing wells. The bottom six areas are vehicle (DMSO) only-containing wells. More than three individual experiments were used for the measurement.

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### 3.3 Effect of melatonin and ramelteon at sub-MIC concentrations on *Porphyromonas gingivalis* gingipain activities

The activities of Rgp and Kgp by melatonin at sub-MIC concentrations were measured. Melatonin inhibited Rgp and Kgp activities in time-dependent and dose-dependent manners. After a 4-hour incubation, significant inhibition was observed. The inhibition rates by 25 µg/mL melatonin on Rgp and Kgp activities were 27.7% and 33.8%, respectively (Fig 4B and 4D). The inhibition rates of 50 µg/mL melatonin on Rgp and Kgp activities were 24.5% and 37.6%, respectively. The inhibitory effects within 4 hours are also shown in Fig 4A and 4C. The activities of Rgp and Kgp were also inhibited by ramelteon at sub-MIC concentrations ( $p < 0.001$ ). As shown in Fig 4F and 4H, the inhibition rates of 12.5 µg/mL ramelteon on Rgp and Kgp activities were 37.3% and 45.2%, respectively. The inhibition rates of 25 µg/mL ramelteon on Rgp and Kgp activities were 23.8% and 37.8%, respectively. The inhibitory effects within 4 hours are also shown in Fig 4E and 4G.

### 3.4 Effect of melatonin and ramelteon at sub-MIC concentrations on hemolytic activity

Because lysed erythrocytes by bacteria could release hemoglobin, the absorbance of 405 nm for red pigment was used to describe the hemolysis of erythrocytes. After a 4-hour incubation of a mixture of *Porphyromonas gingivalis* cells and 1% sheep erythrocytes, hemolytic activity of *Porphyromonas gingivalis* was suppressed by melatonin and ramelteon at sub-MIC concentrations. As shown in Fig 5A, the relative hemolytic activities of melatonin (25 and 50 µg/mL) were 86.32±2.63% and 68.96±5.44%, respectively, compared to the control. The relative hemolytic activities of ramelteon (12.5 and 25 µg/mL) were 76.57±2.28% and 66.56±3.36%, respectively, compared to the control's activity (Fig 5B). The results indicated that melatonin and ramelteon at sub-MIC concentrations inhibited the hemolytic activity of *Porphyromonas gingivalis*.

3.5 Effect of melatonin and ramelteon at sub-MIC concentrations on mRNA expression of virulence factors in *Porphyromonas gingivalis* The transcriptomic responses of virulence factors to melatonin and ramelteon were evaluated using real-time PCR analysis. As shown in Fig 6A, the expression of virulence factors,

Fig 2. Effect of melatonin and ramelteon on *Porphyromonas gingivalis* biofilms formation. *Porphyromonas gingivalis* were incubated in the presence of melatonin (A) and ramelteon (B). After crystal violet staining, absorbance at 550 nm was recorded. Data represent the mean±SEM (n = 3) and differences between groups were assessed by ANOVA,\*P<0.05 versus vehicle control;\*\*P<0.01 versus vehicle control. three experiments.

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including *rgpA*, *rgpB*, *kgp*, *hagA*, and *dragA*, was noticeably inhibited, while the expression of *ftn*, which encodes an iron/heme utilization-related protein, significantly increased in the presence of 50µg/mL melatonin. In the presence of 25µg/mL ramelteon, the expression of virulence factors, including *rgpA*, *rgpB*, *kgp*, *hagA*, *ragA*, and *vimA*, also significantly decreased, while the expression of *hem*, which encodes an iron/heme utilization-related protein, significantly increased (Fig 6B). The expression of *ohmuR*, which encodes a heme iron metabolism-related protein, did not significantly change, although it did show a non-significant, slight increase. The results indicate that melatonin and ramelteon inhibited the expression of virulence factors and affected the expression of iron/heme utilization-related genes in *Porphyromonas gingivalis*.

Fig 3. CLSM observation of *Porphyromonas gingivalis* biofilms formed in the presence of melatonin and ramelteon at sub-MIC concentrations. (A) CLSM images of *Porphyromonas gingivalis* biofilm formed in the presence of melatonin and ramelteon at sub-MIC concentrations. Biofilm-forming cells were stained using the Live/Dead Bacterial Viability Kit. Dead cells were stained red, whereas live bacteria were stained green. In the presence of 50µg/mL or 25µg/mL ramelteon, the areas of biofilm formation were narrower than that of vehicle controls. Bars = 50µm. (B) Effects of melatonin and ramelteon on the thickness of *Porphyromonas gingivalis* biofilms. Data represent the mean±SEM (n = 12) and differences between groups were assessed by ANOVA,\*\*P<0.01 versus vehicle control.

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Fig 4. Effect of melatonin and ramelteon at sub-MIC concentrations on *Porphyromonas gingivalis* Rgp and Kgp activities. A 100% value was assigned to the degradation obtained after a 4 h-treatment in the absence of drugs. A, B, E, and F show the Rgp activities. C, D, G, and H show the Kgp activities. The inhibition of substrate degradation was also measured as a function of time (B, D, F, H). Data represent the mean±SEM (n = 3) and differences between groups were assessed by ANOVA,\*\*P<0.01 versus vehicle control.

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3.6 Cell viability and anti-inflammation of melatonin and ramelteon on HGFs The cytotoxicity of melatonin and ramelteon on HGFs was investigated using MTT. As shown in Fig 7, there were no obvious cytotoxic effects with a 24 h treatment with melatonin.

Similarly, there were no obvious cytotoxic effects following a 24 h treatment with up to 200µg/mL ramelteon. As shown in Fig 8, the releases of IL-6 and IL-8 were stimulated by Pg-LPS after 24 h of exposure, 531.6±38.5 pg/mL and 156.1±23.5 pg/mL, respectively. Melatonin at the sub-MIC concentration (25µg/mL) inhibited Pg-LPS-stimulated IL-6 and IL-8 production, 327.1±25.7 pg/mL and 72.2±11.7 pg/mL, respectively. Similarly, ramelteon at the sub-MIC concentration (12.5µg/mL) also inhibited IL-6 and IL-8 production to a great extent, 296.4±20.7 pg/mL and 69.3±13.3 pg/mL, respectively. Furthermore, the protective effects of melatonin and ramelteon were reversed by the melatonin receptor antagonist luzindole (100 nM) (Fig 8). The above

Fig 5. Effect of melatonin and ramelteon at sub-MIC concentrations on *Porphyromonas gingivalis* hemolytic activity. As a negative control, erythrocytes were used alone. Relative hemolytic activity was determined compared to the vehicle group. The hemolytic activity of vehicle-treated *Porphyromonas gingivalis* was normalized as 100%, and the melatonin-treated group (A) and melatonin-treated group (B) reported here are shown as a percentage of the vehicle control. Data represent the mean±SEM (n = 6) and differences between groups were assessed by ANOVA, \*\*P<0.01 versus vehicle control.

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Fig 6. Effect of melatonin and ramelteon at sub-MIC concentrations on mRNA expression of virulence factors and iron/heme utilization-related genes in *Porphyromonas gingivalis*. Bacteria were incubated in the presence of 50µg/mL melatonin (A) and 25µg/mL ramelteon (B). Data are expressed as the mean±SEM (n = 4) and differences between groups were assessed by ANOVA. The expression was normalized to galE. \*P<0.05 versus vehicle control. \*\*P<0.01 versus vehicle control.

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data demonstrated that melatonin receptor agonists inhibited Pg-LPS-stimulated cytokines expression, which was reversed by the melatonin receptor antagonist.

Discussion This study reports the effects of melatonin and melatonin receptor agonist on the key periodontal pathogen *Porphyromonas gingivalis* for the first time. We show that they have in vitro antimicrobial activity against *Porphyromonas gingivalis*, including the inhibition of biofilm formation, reduction of established biofilms, suppression of Kgp and Rgp, and inhibition of hemolytic activity. The effects may be associated with the regulation of virulence factor genes and iron/heme utilization-related genes. In the meantime, they also exert anti-inflammation from PG-LPS-stimulated cytokines release via melatonin receptor. These data preliminarily indicate that melatonin receptor agonists may be as novel “perioceutics” agents that effectively exert antimicrobial activity and anti-inflammation. Oral bacteria are associated with highly organized microbial communities in the form of dental plaques [42]. As far as most oral pathogenic species are concerned, one of the most key

Fig 7. Effect of melatonin and ramelteon on HGFs viability. Treatment with melatonin has no obvious cytotoxicity on HGFs using MTT. Treatment with ramelteon up to 200µg/mL has no significant cytotoxicity on HGFs. The cell viability obtained in the presence of the vehicle was assigned a value of 100%. Data represent the mean±SEM (n = 4) and differences between groups were assessed by ANOVA, \*\*P<0.01 versus vehicle control.

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virulence factors is the ability to form biofilms. Bacteria within a biofilm are less sensitive to antimicrobial agents than their planktonic counterparts and exhibit insensitivity to host defense systems [43–45]. So, we studied the effects of melatonin and ramelteon on *Porphyromonas gingivalis* biofilms, based on MIC and MBC results suggesting that they inhibit planktonic bacteria. Furthermore, both melatonin and ramelteon show inhibitory effects on *Porphyromonas gingivalis* biofilm formation and established biofilms. The inhibition of established biofilm formation may largely contribute to the reduction of bacterial growth and increased cell death. Another explanation may be due to the damage of the organized microstructure in *Porphyromonas gingivalis* biofilms because melatonin and ramelteon are of high lipophilicity and the ability to enter membranes, as disintegrated biofilms are more vulnerable to drug treatment. It is well known that Rgp and Kgp are predominant extracellular proteolytic enzymes and key virulence factors of *Porphyromonas gingivalis* [46]. They play a pivotal role in the pathogenic factors of *Porphyromonas gingivalis*, for example fimbriae assembly and the processing of outer membrane proteins, and degradation of host proteins. Some of host proteins are thoroughly digested into peptides for furnishing *Porphyromonas gingivalis* growth and metabolism. The others are proteolyzed limitedly or selectively, resulting in the impairment of host immune defense and incapable of avoiding *Porphyromonas gingivalis* [34, 47–49]. So, as far as the above factors are concerned, the suppression of bacterial gingipains by melatonin and its derivative may benefit their potential application in the therapy of periodontitis. Our data

Fig 8. Effect of melatonin and ramelteon on the levels of IL-6 (A) and IL-8 (B) in HGFs. HGFs were treated with melatonin receptor agonists for 6 h, followed by Pg-LPS at 1 µg/mL concentration for 24 h. Data are expressed as the mean ± SEM (n = 4) and differences between groups were determined by ANOVA. \*\*P < 0.01 versus control. #P < 0.05 versus Pg-LPS group, ##P < 0.05 versus Pg-LPS group.

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showed that sub-MIC concentrations of melatonin and ramelteon could significantly inhibit the hydrolytic activities of *Porphyromonas gingivalis* gingipains. The inhibition of gingipains by melatonin and ramelteon will also deprive *Porphyromonas gingivalis* of available iron and prevent some tissue destruction. Thus, it suggested that these substances may be beneficial to reduce periodontal tissue destruction through the proteinase activity of *Porphyromonas gingivalis*. Besides a broad spectrum of proteolytic enzymes in vitro, *Porphyromonas gingivalis* also produces hemolysin, which functions in the release of heme from erythrocytes. These enzymes likely degrade host tissues and secretions for the metabolism [38]. The release of heme from the hemoglobin molecule during the in vivo course of inflammatory periodontal disease may be a major consequence of this disease, and it may control the growth of *Porphyromonas gingivalis* in the subgingival environment [50]. It demonstrated that *Porphyromonas gingivalis* hemolysin is capable of degrading the hemoglobin molecule and eventually transporting it into the cell [51]. The mechanism of heme acquisition from erythrocytes involves hemagglutination, hemolysis, binding, and degradation of the hemoglobin molecule [51]. Due to its high metal binding

capacity, melatonin is assumed to function differently from known antimicrobials by reducing intracellular substrates in microorganisms [52]. In our study, melatonin and ramelteon inhibited hemolytic activity and may be associated with metal binding. In addition, genes involved in virulence of *Porphyromonas gingivalis* were affected by treatment with melatonin and ramelteon. Melatonin and ramelteon inhibited the expression of *kgp*, *rgpB*, *vimA*, *ragA*, and *hagA*, while they increased the expression of *fhmuR*, *luxS*, *ftn*, and *hem*. *HagA* is considered to be a key virulence factor because of the acquisition of heme from erythrocytes and other host cells, necessarily for bacterial growth [53–55]. *HagA* is also involved in attachment to human coronary artery endothelial cells and gingival epithelial cells [53–55]. *RagA*, which is the immunodominant surface antigen in the serum of patients with periodontal disease, is stimulated when *Porphyromonas gingivalis* is treated with cotinine, nicotine, and cigarette smoke extract. *Rag* is the homolog of TonB-linked outer membrane receptors and involved in the acquisition of iron in *Porphyromonas gingivalis* [55–57]. *Rgp B* plays a critical role in the periodontal disease because it is in charge of the production of gingipains [55,58,59]. Consistent with the above result, the above virulence-related genes were suppressed by melatonin and ramelteon, which may play a role in their antimicrobial and antiprotease activities. In our study, the gene *ftn* was up-regulated by melatonin and *hem* by ramelteon. Although there were not significant differences in the expression levels of *fhmuR* and *luxS*, the drugs had a tendency to increase the expression levels of genes related to iron/heme utilization. Indeed, there exists a relationship between iron/heme uptake and virulence in *Porphyromonas gingivalis* [60]. However, the virulence factors of *Porphyromonas gingivalis* are very complicated and multifactorial, so the nature of this regulation is still unclear. This inconsistency requires further study. Due to their lipophilic nature, melatonin and ramelteon can easily access to the cell. They directly deposit in the oral cavity so that they have the high concentration to improve the health of oral tissues. One of the desired properties of melatonin receptor agonists is low toxicity to eukaryotic cells. In this study, the MTT assay was conducted with HGFs to assess cytotoxicity. Melatonin had no obvious cytotoxic effects, which was consistent with previous results that examined the proliferation of HGFs and MC3T3-E1 cells [8,16]. Similarly, no obvious cytotoxic effects were observed following a 24h-treatment with up to 200 µg/mL ramelteon. PG-LPS, a component of the gram-negative bacterial cell wall, has been identified as the main virulence factor to induce periodontal disease and in correlation with the severity of

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periodontal disease [61,62]. It can stimulate the production of interleukins (IL), such as IL-6 and IL-8, in HGFs [26,63–65]. IL-6 is the inflammatory cytokine produced after microbial recognition and is involved in the pathogenesis periodontal disease, such as osteoclast formation, bone resorption, and periodontal destruction. IL-8 can recruit neutrophils to the periodontal lesion as a major chemoattractant. These ILs promote the degeneration of inflamed periodontal tissues and inhibition of them should be beneficial to treat periodontal disease [65,66]. In the present study, melatonin and ramelteon at sub-MIC concentration were both found to inhibit IL-6 and IL-8 production. The anti-inflammatory action is partly through melatonin receptors because this action was reversed by the melatonin receptor antagonist luzindole. It has been proved anti-inflammatory action of melatonin is thought as a direct result of potent radical-scavenging ability via the MT

receptor [67,68]. It has also been reported that ramelteon reduced nicotinamide adenine dinucleotide phosphate (NADPH) partly mediated by MT receptor, which is involved in oxidative stress [69]. Based on these reports, we believe that anti-inflammation of melatonin and ramelteon may have involved an antioxidant effect partly caused by MT receptor. In conclusion, this study firstly showed that melatonin and ramelteon have in vitro antimicrobial activity against both the planktonic culture and biofilms of *Porphyromonas gingivalis*. They inhibit the proteinase activities of gingipain and hemolytic activity, which are associated with the expression of virulence factors and iron/heme utilization. Furthermore, they exhibit anti-inflammatory properties via melatonin receptors. Our study provides new evidence that melatonin receptor agonists might be useful as the novel “Perioceutics” agents in periodontal disease therapy.

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#### **Author Contributions**

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Investigation: WZ XZ JPL ZCS.

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Validation: WZ XZ.

Writing – original draft: WZ XZ JPL ZCS.

Writing – review & editing: WZ XZ JPL ZCS.

38. Which is NOT a natural way for you to increase your healthy levels of melatonin?

- a. Eat foods rich in tryptophan
- b. Sleep between 7 to 9 hours a night
- c. Stop drinking caffeine or eating caffeine-rich foods after 2 PM
- d. Perform aerobic exercise just before bed

Dietary factors and fluctuating levels of melatonin

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Abstract

Melatonin is secreted principally by the pineal gland and mainly at nighttime. The primary physiological function is to convey information of the daily cycle of light and darkness to the body. In addition, it may have other health-related functions. Melatonin is synthesized from tryptophan, an essential dietary amino acid. It has been demonstrated that some nutritional factors, such as intake of vegetables, caffeine, and some vitamins and minerals, could modify melatonin production but with less intensity than light, the most dominant synchronizer of melatonin production. This review will focus on the nutritional factors apart from the intake of tryptophan that affect melatonin levels in humans. Overall, foods containing melatonin or promoting the synthesis of it by impacting the availability of tryptophan, as well those containing vitamins and minerals which are needed as co-factors and activators in the synthesis of melatonin, may modulate the levels of melatonin. Even so, the influence of daytime diet on the synthesis of nocturnal melatonin is limited, however, the influence of the diet seems to be more obvious on the daytime levels.

Keywords: melatonin; 6-sulphatoxymelatonin; diet; vegetable; alcohol; B vitamins

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July 2012 Many normal physiological functions occur at specific times of the day. These biological rhythms, called circadian rhythms, are controlled by an endogenous time-keeping system oscillating approximately 24-h cycle under constant conditions (1, 2). The master oscillator, which responds to interior and exterior signals, is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus in the brain. The most powerful pacemaker is the environmental light-dark cycle. SCN neurons transmit this rhythmic light-dark information to other brain regions and peripheral organs that control many aspects of physiology and behavior, including sleep-wake cycles, cardiovascular activity, the endocrine system, dietary behavior and energy metabolism. These signals are distributed via two main pathways: the direct pathway, which uses the autonomous nervous system and the indirect pathway, which operates via hormonal signals controlled by the SCN, such as the nocturnal peaking secretion of pineal melatonin or the daily increase in adrenal glucocorticoids (3). Disruption of the circadian rhythm and sleep-wake cycles are considered risk factors for a variety of health problems including obesity and cardiovascular disease (3, 4). Several preclinical studies have identified dietary components, such as glucose (5, 6), sodium (7), ethanol (8), or caffeine (9) being capable of phase-shifting circadian rhythms by modifying the expression of genetic components of the biological clock, i.e. clock genes. Changes in the circadian cycle modify metabolism, but in addition, alterations in metabolism are also able to entrain physiological clocks, resulting in

changes to the rhythms as outlined previously (1, 4). One possible mechanism by which diet can influence circadian rhythms is by modifying the secretion of melatonin. Melatonin is a circulating neurohormone secreted predominantly at night. It is important in conveying the daily cycle of light and darkness to the body, thus regulating circadian rhythms and helping us to fall and stay asleep. In addition to its' regulatory role, melatonin has antioxidative capacity, immunomodulatory potency, and also appears to be protective against a variety of cancers, especially breast cancer, although data is based mostly on observational studies and animal models (10–12). Exogenous melatonin has been used for the treatment of sleep disorders of circadian origin such as jet lag and delayed sleep phase syndrome and as complement of other therapeutic drugs for the treatment of numerous diseases including glaucoma, irritable bowel disease, and certain types of cancers (page number not for citation purpose)

#### Review Article

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mainly to either enhance the therapeutic effect of conventional drug therapy or to reduce their toxicity thus ameliorating the side-effects (13–18). Negative side-effects of exogenous melatonin in abovementioned clinical trials are rare. The regulating system for the secretion of melatonin is complex. Light is the most influential environmental factor. In addition, melatonin synthesis depends upon availability of its precursor, tryptophan (TRP), which is an essential amino acid and is thus an essential component of the diet. If intake of TRP is severely restricted, synthesis of melatonin is significantly reduced in humans (19). Few studies have explored the influence of other dietary factors on the fluctuation of melatonin levels, for example the intake of certain food items or the availability of nutrients. This narrative review will focus on the nutritional factors apart from the intake of TRP that affect melatonin levels in humans. We used the electronic bibliographical database PubMed until April 2012 (without any methodological restrictions), to identify studies using the following keywords: melatonin, 6-sulphatoxymelatonin, diet, vegetable, alcohol, and B vitamins. In addition, we reviewed the references of identified studies and of selected narrative review articles.

**Synthesis of melatonin** The synthesis and physiological function of melatonin have been recently described accurately in several review articles and are here only very briefly summarized (10, 20–25). Melatonin is a neurotransmitter secreted predominantly by the pineal gland. There are extra-pineal sites of melatonin production, such as the retina and the gut. Even if the synthesis of melatonin in these extra-pineal sites contribute to the total concentration of melatonin, the physiological significance of these sites is uncertain. Melatonin is synthesized from its precursor, the essential amino acid TRP (Fig. 1). The rate of melatonin formation depends on the activity of enzymes arylalkylamine N-acetyltransferase (AANAT) and to a lesser extent, tryptophan hydroxylase (TPH). AANAT is represented mostly in the pineal gland where its activity is controlled by SCN. Activity of pineal

TPH fluctuates in a clock-driven circadian rhythm, which reaches its highest levels during the night. Melatonin synthesis is controlled by both an endogenous circadian clock and by environmental light. Light is the dominant environmental factor that controls its synthesis. Pineal melatonin levels begin increasing in the

Fig. 1. The synthetic pathway and metabolism of melatonin and possible sites for some nutrients to influence on the synthesis. Enzymes are in underlined capital letters in italics. TRP, tryptophan; TPH, tryptophan hydroxylase; TDO, tryptophan dioxygenase; AADC, aromatic aminoacid decarboxylase; AANAT, arylalkylamone-N-acetyltransferase; HIOMT, hydroxyindole-O-methyltransferase; 6-SMT, 6-sulphatoxymelatonin; SAME, S-adenosylmethionine.

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late evening, reaching the maximum in the early hours between 2:00 and 4:00 a.m., followed by a slow decline to lower daytime levels. Daytime levels of melatonin are barely detectable. In addition to sunlight, artificial indoor lighting can be bright enough to prevent the nocturnal release of melatonin. Normal production of melatonin can vary considerably between individuals. In general, melatonin production decrease with aging. Among the other factors that have been most consistently linked to modified melatonin levels are disrupted light dark cycles, night work and being overweight. In addition, it has been demonstrated that some nutritional factors could also modify melatonin production, however less intensely. Weather dietary factors contribute only to the pineal production of melatonin or also to the extra-pineal sites such as the gastrointestinal production is not known. Once synthesized, melatonin is not stored in producing cells, but is quickly released into the blood and begins circulation. Thus, the concentration in plasma faithfully reflects pineal secretion. In addition melatonin concentration can be measured in other body fluids as saliva and urine. Saliva levels are about 40% of those in plasma. Endogenous nocturnal melatonin production has been estimated to be about 10–80 mg per night, the daytime production being significantly less. The metabolism of melatonin is rapid, and its half-life in humans varies between 10 and 60 min following exogenous administration. It is deactivated mostly by the liver and excreted in urine. Urinary metabolite, 6-sulphatoxymelatonin (6-SMT) reflects the plasma melatonin profile and can be used for evaluation of melatonin status. In urine, 50–80% of 6-SMT appears in the overnight sample.

Dietary compounds There are several studies indicating dietary influence on the synthesis or the concentration of melatonin.

Energy restriction Strong influence of food on melatonin synthesis is detected in studies of subjects undergoing periods of fasting. Energy restriction reduces the nocturnal secretion of melatonin although the number of human studies proving this is limited. Short-term voluntary fasting by total rejection of food or with very limited intake of energy (B300 kcal per day) from 2 to 7 days reduces melatonin concentration in the blood by about 20% (26–28). In these studies, however, no changes were noticed in the excreted metabolites of melatonin in urine. Glucose supplementation during short-term fasting returns the decreased melatonin concentration to normal, suggesting that human pinealocytes, or

other producing cells, require a certain minimal amount of glucose delivery to function normally (27, 28).

**Edible plants and plant-based products** Some food items, especially edible plants, contain melatonin and its precursor TRP as well. Actually, the presence of melatonin in plants is universal, although with widely varied concentrations from picograms to micrograms per gram of plant tissue (29). Melatonin has been detected in notable amounts for example in tomatoes, olives, barley, rice and walnuts (Table 1) (29–40). Recent studies have uncovered that melatonin concentrations differ not only among plant species, but also within varieties of the same species. In grapes, for example, cultivars Nebbiolo and Croatina have very high melatonin levels, about 0.8–0.9 ng/g, whereas Cabernet franc contains only 0.005 ng/g. Thus, in a glass of wine, the concentration of melatonin may vary considerably, from picograms to many nanograms per milliliter (41, 42). The bioavailability of plant-based melatonin has been demonstrated mainly in animals but also in humans (30, 35, 43). It has been measured as increased blood concentration or heightened amounts of excreted urinary 6-SMT after the ingestion of melatonin-containing products, such as vegetables or barley-based beer (43, 44). Even some dose dependency has been noticed—the mean urinary 6-SMT was 16% higher in Japanese women within the highest quartile of vegetable intake compared to those with the lowest intake (45). However, vegetables and grain products contain large amounts of vitamins and minerals. Thus, even if increased levels of melatonin have been noticed in these studies, it cannot be excluded that the increase is due to boosted endogenous melatonin synthesis by the possible stimulatory effect of ingested products instead of absorbed dietary melatonin only. In addition to wine, remarkably high melatonin concentrations have been detected in coffee beans as was outlined previously (29, 35). Although a cup of coffee is estimated to contain even as much as 40 mg of melatonin, corresponding the nocturnal endogenous production, the general effect in the circulating melatonin concentration may differ, since coffee contains caffeine which may Table 1. Some examples of melatonin content in plants and foods measured by immunological and chromatographic techniques

Plant/food Melatonin Reference

Tomato	3	114 ng/g	32, 37	Walnuts	3	4 ng/g	30	Cereals (rice, barley)	300–1,000 pg/g	39
Strawberry	1	11 ng/g	32	Olive oil	53	119 pg/ml	34	Wine	50–230 pg/ml	32, 36
	52	170 pg/ml	43	Cow's milk (unprocessed)	3	25 pg/ml	38, 40	Nighttime milk	10–40 ng/ml	62

**Dietary factors and melatonin**

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reduce endogenous nocturnal melatonin levels. Results in clinical studies are conflicting. In two short-term studies, a single dose of 200 mg caffeine capsules decreased nighttime melatonin secretion, whereas a significant increase of 32% was observed in another study (46–48). In still another study, subjects were administered repeated 400 mg doses of caffeine capsules at 1-week intervals. Analysis yielded a slight trend of 7% reduction of nighttime melatonin levels in healthy young adults (49). When caffeine was administered to study subjects via coffee, reduction of more than 50% was found in nighttime 6-SMT excretion compared with decaffeinated coffee in another small study (50). Caffeine has both the stimulatory and inhibitory mechanisms affecting the levels of melatonin. Which of these dominate in normal healthy subjects is not clear since hypothesis of mechanisms

have been tested mostly in vitro and by using animal models. Caffeine may alter the expression level of clock genes either up-regulating or down-regulating clock gene amplitudes (51, 52). Caffeine acts as an adenosine receptor antagonist. Adenosine increases intracellular cAMP levels via adenosine receptor, which increases the production of AANAT, the rate limiting enzyme in melatonin synthesis (53). Thus as adenosine receptors are blocked by caffeine, the synthesis of melatonin decreases. In addition caffeine may also reduce the break-down of melatonin. Caffeine and melatonin compete for the same metabolizing liver cytochrome P450 enzymes, resulting in higher serum levels of melatonin after large doses of caffeine (48, 54). There are significant differences in before mentioned studies in their designs, in timing and dosing of caffeine as well as sampling and analyzing melatonin and its metabolite. In addition, subjects were either sleepdeprived or not, which greatly modify synthesis of melatonin and can at least partly explain the differences. If study subjects include both males and females, it may also have an influence on the results, since menstruation has been reported to modify such levels, while oral contraceptives have been found to increase nighttime melatonin levels due to inhibiting catalyzing enzymes in the liver (55–58).

Nighttime milk Melatonin is a natural compound found in milk. Concentration of melatonin exhibits a marked daily rhythm, with increasing concentrations in milk produced at night (40, 59). This phenomenon seems to be universal amongst mammals. Since milk is the primary component of an infant's diet, and nighttime lactation confirms the nutritional adequacy, melatonin content of nighttime milk may have further physiological relevance. This theory may benefit from further study, but it seems plausible that maternal melatonin may pass through milk to the infant resulting in improved nocturnal sleep, although this is based on a very limited number of observations (59). In addition, if TRP-enriched commercial infant milk formula is served at night, improvements in sleep parameters and increased urinary metabolites of serotonin were observed, suggesting increased use of serotonin to produce melatonin (60, 61). Studies showing the influence of the melatonin content of cow's milk on the fluctuation of melatonin levels in adults are even scarcer. In institutionalized elderly subjects, commercial milk was replaced with melatonin-rich nighttime milk in a double-blind study (62). The ingested total amount of 10–20 ng melatonin from nighttime milk is, however, such a small dose divided into several meals, that physiological significance of it is hard to understand. Morning activity of subjects, however, was significantly increased, indicating some difference between normal commercial milk and nighttime milk, of which the content of melatonin is most obvious.

Alcohol Conclusions regarding the influence of alcohol on melatonin levels have been found to be inconsistent. Both acute and chronic consumption of alcohol at a level corresponding to 'social drinking' (10–100 g of ethanol per day) reduce melatonin levels in the blood and in the saliva in three small studies of healthy volunteers (63–65), but not in a fourth study (66). In addition to absolute amount of ethanol, other properties of alcoholic beverages may also have an influence on the overall effect. As mentioned above, wine and beer contain melatonin, and consequently may have additional influence on detectable melatonin levels in the body. In a study of strong beer (alcoholic content of 7.2%), 45 min after a single dose of 330 ml for women and 660 ml for men, corresponding doses of 24–48 g of ethanol, melatonin level in serum was significantly elevated in a small study with seven healthy subjects (43). This increase was explained by the researchers by the melatonin content of beer. The influence of alcohol on urinary metabolites of melatonin, however,

conflicts with these findings. Even if single or repeated doses of alcohol in the evening reduces the nocturnal melatonin secretion to the blood dose-dependently, no corresponding changes in 9–12 h urinary excretion of melatonin metabolites were noticed (63, 64, 67). In these studies healthy participants—males and females—received doses varying from 15–120 g ethanol per day either as single or repeated daily doses. However, in a large study with over 200 healthy women between the ages of 20 and 74, it was found that the nocturnal urinary concentration of the 6-SMT decreased dose-dependently with increasing consumption of alcoholic beverages in the preceding 24-h period (68). A categorical analysis

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revealed no effect of one drink (on over 70% of observed days), but a 9% reduction with two drinks, a 15% reduction with three drinks, and a 17% reduction with four or more drinks (on only 2% of observed days). This was shown also after taking into account the effects of age, number of daily hours of darkness, use of medications that affect melatonin levels and body mass index. Thus, alcohol consumption seems to reduce the melatonin concentration in the blood, but whether there is an association between urinary melatonin metabolites and alcohol is not as clear, perhaps due to varied beverages and doses used and differences in methods for urine collection as well as ethanol-based metabolic differences. Several possibilities have been hypothesized to explain how ethanol inhibits melatonin secretion. Hypotheses vary from disturbed sleep patterns and loss of daily rhythm caused by alcohol use to reduced TPH activity to phase delay in AANAT gene expression and activity to disturbed steps in the synthesis or excretion of melatonin (64, 69). In addition, ethanol decreases TRP levels in plasma resulting in a short-term decrease in the availability of TRP. A small amount of glucose is needed in the synthesis of melatonin. Since ethanol has properties that decrease blood-glucose levels, it has been speculated that an insufficient amount of glucose in pinealocytes would explain the reduced levels of melatonin. The mechanisms behind these remain to be studied further.

Availability of nutrients Evidence of nutrients' influence on melatonin synthesis tends to vary. There are some studies, based mostly on animal evidence, which prove the importance of B vitamins, magnesium, zinc and polyunsaturated fatty acids. Folate, magnesium and zinc deficiencies have been linked with lower melatonin levels in rodents and in one rodent study, vitamin B6, either alone or in combination with zinc, increased plasma melatonin (70–73). Folate and B6 vitamin are supposed to boost the formation of serotonin from TRP as coenzymes. Zinc and magnesium, instead, are supposed to enhance the formation of melatonin from serotonin by binding to AANAT enzyme, thus activating it and increasing the affinity of serotonin for binding to AANAT (74, 75). In humans, the role of these vitamins and minerals is less well studied in this connection. In a large study of nearly 300 Japanese women, folate intake was not connected with urinary excretion of 6SMT levels (45). Urinary 6-SMT, however, associated positively clearly with intake of all vegetables and especially with green and yellow vegetables. In a Nurses' Health Study of about 1,000 US women, no association was found between the high intake of various nutrients, such as folate, vitamin B6 and zinc and increased urinary

melatonin excretion (76). Besides marginal positive associations between orange juice, tomato and dairy cream consumption and urinary 6-SMT, only meat consumption was significantly and inversely associated with urinary 6-SMT in this large study. Neither of these studies, however, reported the vitamin status of the subjects, only their calculated dietary intake. In a small study of 12 healthy men, orally-supplemented pyridoxine (one vitamin of vitamin B6) as a dose of 100 mg in the evening hours had no effect on nocturnal melatonin levels in the blood (77). Nocturnal melatonin levels in the blood, however, doubled in over 100 small children between the ages of 18 months and 8 years when pyridoxine was administered at night intravenously at doses of 3 mg/kg (78). Any possible deficiency of pyridoxine in these hospitalized children was not reported. Reliable clinical evidence of boosted melatonin secretion following the ingestion of calcium, magnesium or zinc, does not yet exist. In a study of 10 healthy men, a single intravenous dose of magnesium sulphate was found to have no effect on the release of melatonin (79). No correlation, however, between serum magnesium and melatonin levels was found in patients with intervertebral disc herniation. Instead, a clear positive correlation between serum zinc and melatonin level was found (80). The pineal gland contains high levels of n-6 and n-3 polyunsaturated fatty acids (PUFA), especially arachidonic acid and docosahexaenoic acid (DHA) (81). There is some evidence that fatty acid status can influence melatonin synthesis. In rodents, an n-3 deficient diet reduces nighttime melatonin secretion, which returns to normal with supplemented DHA (82, 83). Based on animal experiments, the rhythm of AANAT activity may be altered by n-3 status and n-3/n-6 ratio, as well as these may modify the activities of membrane-bound proteins including enzymes, receptors and transporter proteins (81). We did not find any similar PUFA studies with humans. The only clinical study to measure the importance of dietary PUFA on melatonin levels in humans as yet found is a study of insomnia patients (84). Subjects ingested dietary supplements in capsule form containing soy oil, cod liver oil, humulus lupulus extract and soy lecithin daily for a month immediately before sleep. Neither this supplement nor placebo capsules containing olive oil had any influence on urinary excretion of 6-SMT (84).

Clinical relevance and conclusions Melatonin secretion is strongly related to the duration of darkness. Its most definitive physiological role is to convey information to the body about day length for a variety of physiological functions. In addition to melatonin's role as an endogenous synchronizer, growing

Dietary factors and melatonin

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evidence suggests its anti-oxidative activity as well as its having a role in modulating immune responses. At present, a growing interest is focused on the validity of the anti-tumor mechanisms of melatonin. Synthesis of melatonin requires tryptophan as a precursor as well as a smoothly functioning cascade of several enzyme-based reactions, first to compose serotonin and subsequently melatonin. Several vitamins and minerals act as co-factors and activators in these processes, thus a clear deficiency of needed nutrients may restrict the synthesis. Severe deficiencies, however, are rare in western countries apart from some subgroups. In addition, fluctuating melatonin levels can be boosted by ingesting products containing melatonin. The bioavailability of plant-based melatonin is evident, at least in rodents, and could explain some health benefits of vegetables, fruits and

grain products. Diet and nutrients modulate fluctuating melatonin levels, but the influence is minor if compared with the power of the light-dark cycle. Other health-related lifestyle factors such as body weight, which is connected to diet, may have as much effect on melatonin levels as specific dietary choices. Thus, the health benefits of diet-driven melatonin boosts seem not to be the product of any single food or nutrients present in the diet. In general, diets rich in vegetables, fruits and grain products contain considerable levels of dietary melatonin. Vitamins and minerals contribute to the synthesis of endogenous melatonin while the body is active. Even so, the influence of daytime diet on the synthesis of nocturnal melatonin is very limited, however, the influence of diet seems to be more obvious on the daytime levels of melatonin.

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**Your Complete Guide on How to Increase Melatonin** Melatonin. You might've heard the word before, you've might've seen the little bottles of it at your local drugstore, or maybe you've even taken it. But do you know what it is and how it works? If you don't you might be using it wrong.

Melatonin is a naturally occurring chemical in our body that makes us sleepy but due to poor nighttime rituals and excessive exposure to certain lights, many of us are hindering its production. If you have trouble sleeping and would like to learn more about how to increase melatonin levels naturally, read on to find out more. **What Is Melatonin** Melatonin is a hormone that our body naturally produces when the sun starts to go down. It's produced in our pineal gland, the part of our brain that controls sleep, and most recently also discovered in our gut.

There aren't a lot of studies to show how much of this sleeping chemical each person produces exactly but overall, experts have agreed that the amount you produce is not nearly as important as when you produce.

Melatonin is present in our bodies throughout the day but when it gets dark, its production will drastically increase. And once produced, the chemical will be distributed throughout our bloodstream. Imagine a robot running around your body telling all the different parts it's time to shut down for the day. **Benefits of Melatonin** Other than restful sleep, melatonin offers a variety of other benefits: • More energy • Better concentration • Prevents memory loss • Keeps immune system strong • Keeps eyes healthy • Gets rid of free radicals • Slows down signs of aging **Immunity** It's not a secret that when you sleep better, your immune system also works better. Overall, sleep promotes the health of all of our organs and especially our body's ability to heal and fight off germs and infections. There's a reason why your doctor tells you to make sure you get a lot of rest and sleep when you're sick. **Cardiovascular Health** Studies show melatonin contains antioxidants, anti-inflammatory, and anti-hypertensive effect, which all help decrease the risk of high blood pressure, heart attacks, and strokes. **Gut Health** Melatonin promotes gut health. As we've mentioned before, this chemical also comes from our gut and part of the reason why... it aids digestion. People with irritable bowel syndrome, acid reflux, or other

common stomach issues can all benefit from it. Antioxidants Melatonin contains high levels of antioxidants which is what we need to counter the effects of free radicals, a damaging atom that we get from “some dirty fried foods, alcohol, tobacco smoke, pesticides, air pollutants, and many more.” Antioxidants can reduce and neutralize the “oxidation damage” that free radicals cause in our cells. Other Benefits Other reported benefits include pain management, PMS, eye health, thyroid functions, memory health and much more. What Affects Melatonin Production People have trouble sleeping for a variety of reasons but one of the most common ones is due to disruptions in our circadian rhythm, which controls the timing of our sleep. These disruptions are caused by a combination of natural and artificial reasons. Too Much Artificial Light Artificial lights simulate natural light, so if you have a lot of bright lights in your house especially your bedroom, it can confuse your body. Our eyes cannot tell the difference between natural lights from artificial lights and may be sending our brains signals that it’s still daytime when really, it’s night time. This disrupts the melatonin secretion in our brain. Technology Your phone, computer, and television screen all contain LED lights, which are sometimes called “blue lights.” These blue lights are known to interrupt sleep.

If you stare at technology too much at night, it affects your body’s circadian rhythm. If the rhythm is messed up, your body won’t know when to increase or decrease melatonin production, which is why you’ll have trouble falling asleep and waking up. Not Getting Enough Sun During the Day In order to naturally increase melatonin, your body needs a certain amount of natural sunlight. This is because sunlight makes serotonin, which is the precursor of melatonin. Once the sun goes down, leftover serotonin converts into melatonin, which is why you need ample sunlight during the day to make sure enough serotonin was created. Seasonal Changes During the fall and winter months, there’s less light, people tend to produce high levels of melatonin. You might’ve noticed yourself that it’s harder to get out of bed during the winter. However, during the time when the seasons change, your body might have a hard time adjusting to the different levels of light. For example, if where you live is transitioning from the summer to the fall, you might find yourself staying up way past bedtime in the fall because your body hasn’t adjusted to the longer hours of darkness yet. Too Much Caffeine This probably doesn’t come as surprise to many, but caffeine suppresses melatonin, which is why it helps people wake up in the morning.

Maybe you don’t think a cup of coffee a day will affect you that much but over time, you are training your body to produce less melatonin and ignore sleepiness, which can lead to chronic fatigue and bigger sleeping problems. How to Increase Melatonin Naturally There are many things one can do to naturally increase melatonin levels without supplements. The biggest one has to do with lights. 1. Take A Break From Technology The best way to repair your circadian rhythm, experts say, is to stop using technology for a prolonged period of time. One week was found to be the perfect amount of time to normalize sleeping patterns for a group of participants who were asked to go camping for a week. You can try to not touch any technology at home for a week but let’s face it, in today’s world it is nearly impossible to avoid technology at home. Even if you don’t want to watch TV, your spouse or your children might and that’ll tempt you to watch too.

The best solution is to plan a getaway for the whole family and turn off all the technology. If you must use it, designate only one or two hours in the middle of the day for it. Plus, leaving technology behind is a great way for the family to bond face to face. 2. Start Dimming Lights

Early Most people make the mistake of thinking melatonin starts when they turn off the lights to go to sleep, but this is not true. Melatonin levels increase when your body starts to sense there is less light.

What you can do to aid this process is by dimming the lights in your house and bedroom earlier. At least one hour before bedtime, start to turn off the lights in the house that you do not need and only leave on the ones that are crucial. If you only have one light in your bedroom, consider getting a desk lamp or installing a light dimmer so you can control the amount of light you can have in your room. By reducing the intensity of the light in your house way before bedtime, you'll be signaling to your body to get ready for bed and this should help the chemicals going. 3. Reduce Exposure to Blue Lights Before Bed Time We're all guilty of this. Scrolling through our phones before bedtime but this is probably one of the worst sleeping habits anyone can have. The blue light emitted from your phone screen is distinctly harmful to melatonin production.

It's not easy to put away the phone though, we know. What we suggest is for you keep the phone outside of your bedroom. Leave it charging in the kitchen or in the living room and let your friends and family know that you are trying to reduce phone usage before bedtime so they don't call unless it's an emergency. 4. Cut Back on Social Media Similar to point number three, social media is one of the reasons why many people are addicted to their cell phones and computers. If you find yourself scrolling through social media for hours before bedtime, stop.

It's harder said than done but some ways to do this is to turn off notification settings, put the icons in specific folders, or delete the icons completely so you can only check them on a computer. 5. Eat A Healthy Diet While this seems like a generic piece of advice, a healthy diet is crucial to better sleep. In fact, did you know all plants have a certain amount of melatonin in them? That's because plants, like us, also rely on light to grow.

Foods that have a high amount of naturally occurring melatonin are:

- Tart cherries • Asparagus • Tomatoes • Sweet potato • Pomegranate • Olives • Nuts and seeds

Foods that are rich in tryptophan, magnesium, calcium, and B6 are also known to promote sleepiness. 6. Increase Relaxation Another way to induce sleepiness at night is to increase relaxation and this could mean different things for different people. For example, music might relax one person but might stimulate another.

Here's some ideas:

- Take a bath • Essential oil diffuser • Play soothing music • Use a sound machine that plays white noise • Drink non-caffeinated herbal tea • Stretche • Search, Ponder and Pray

What About Melatonin Supplements Supplements can be great if you need an extra boost for reasons like jetlag or seasonal changes. You can use it to help you correct your circadian rhythm but we wouldn't recommend thinking of it as a replacement for your own melatonin.

Your body is naturally able to produce melatonin and if you have trouble sleeping, it probably means its production cycle is off. What you need to do is follow the recommended advice above to try to normalize it. Are Melatonin Supplements Safe? If you do decide to take melatonin, try to take the synthetic kind because the natural kind contains substances from the pineal glands of animals which carries the risks of viruses.

Compared with other sleeping medications, melatonin is considered relatively safe but if you have other health issues and are taking other types of medication, it is best to always

check with your doctor to make sure it won't interfere with any other drugs. Can You Take Too Much? An overdose of melatonin is unlikely but it does have its own side effects. These include:

- excessive drowsiness • headaches • vivid dreams (might not be so bad) • changes in blood pressure

A common thing that people misunderstand about melatonin is that the amount you take does not make you sleepier. So if you take melatonin and it doesn't work for you, taking more won't make it more effective. Doctors say taking 1-3 mg an hour before you sleep should do the trick. Healthy Sleeping Habits Are Crucial Next, to food, sleep is arguably one of the most important things in our lives. Without it, our body starts to shut down, our organs fail, and our responses are delayed.

If you have poor sleeping habits, no amount of melatonin supplements will help you. Good sleep starts with healthy sleeping habits and these habits shouldn't just be in the bedroom. We already learned how to increase melatonin by reducing light exposure before bedtime but remember a wholesome diet is just as important.

Once you've tried all these tips and tricks and you still have trouble sleeping, there might a bigger issue at hand. Consult a medical professional if sleeping problems persist. If you'd like more advice and tips on leading a healthier life, be sure to check out our blog for more!

39. Clifford Consulting and Research provides a test to determine a patient's sensitivities so that the least offensive dental material can be chosen and used for dental treatment.

- a. True
- b. False

What is Clifford Materials Reactivity Testing?

In modern society we come in contact with many substances every day in the environment, the food we eat, the products we use and the treatments we receive.

Because each of us possesses unique chemical sensitivities, these substances affect each of us differently and in varying degrees. For some, the effects of certain substances (and their corrosion byproducts) can be toxic and hazardous, and may result in serious health problems. A substance which causes little or no reaction in one individual can prove harmful to another.

Since these effects may vary in each of us, it is vital that these material sensitivities be considered when choosing dental and other materials for use in the body, especially in patients with rare or unique health concerns.

Clifford Materials Reactivity Testing (CMRT) provides dentists and physicians with extensive information about their patient's individual sensitivities so that least offensive materials can be chosen and used in their treatments

CMRT is not merely based upon the body's response to biomaterials themselves, but also upon response to corrosion byproducts of those materials. These byproducts are generated at various rates as the materials decompose or break down in the body. It is these products of decomposition that the body must deal with and which are most likely to cause untoward health effects and toxic conditions. The body produces systemic antibodies

against challenging antigens and will maintain an immunologic record of the components or chemical families which have proven offensive and which can be observed by CMRT. Clifford Materials Reactivity Testing (CMRT) is a laboratory screening test used to help identify existing sensitivity problems to various chemical groups in an individual patient. This test is currently offered in Dental and Orthopedic panels. The same 94 chemical groups are tested in both panels, but correlated with different databases of products depending on the panel ordered. The Dental panel reports on over 17,204 trade-named dental products. The Orthopedic panel reports on over 10,427 trade-named products for surgical applications.

#### 40. What is NOT a cause of receding gums?

- a. Inflammation from periodontal disease.
- b. Tooth brushing with intact and healthy bone remaining between the facial gingival tissue and the root surface
- c. Occlusal trauma "wiggling" the alveolar bone
- d. Specific anatomical areas exhibiting lack of alveolar bone on the facial of roots since birth

**Receding Gums** Gum recession is the process in which the margin of the gum tissue that surrounds the teeth wears away, or pulls back, exposing more of the tooth, or the tooth's root. When gum recession occurs, "pockets," or gaps, form between the teeth and gum line, making it easy for disease-causing bacteria to build up. If left untreated, the supporting tissue and bone structures of the teeth can be severely damaged, and may ultimately result in tooth loss. Gum recession is a common dental problem. Most people don't know they have gum recession because it occurs gradually. The first sign of gum recession is usually tooth sensitivity, or you may notice a tooth looks longer than normal. Typically, a notch can be felt near the gum line. Gum recession is not something you want to ignore. If you think your gums are receding, make an appointment with your dentist. There are treatments that can repair the gum and prevent further damage.

**Why Do Gums Recede?** There are a number of factors that can cause your gums to recede, including: Periodontal diseases. These are bacterial gum infections that destroy gum tissue and supporting bone that hold your teeth in place. Gum disease is the main cause of gum recession. Your genes. Some people may be more susceptible to gum disease. In fact, studies show that 30% of the population may be predisposed to gum disease, regardless of how well they care for their teeth. Aggressive tooth brushing. If you brush your teeth too hard or the wrong way, it can cause the enamel on your teeth to wear away and your gums to recede. Insufficient dental care. Inadequate brushing, flossing, and rinsing with antibacterial mouthwash makes it easy for plaque to turn into calculus (tartar) -a hard substance that builds on and between your teeth and can only be removed by a professional dental cleaning. It can lead to gum recession. Hormonal changes. Fluctuations in female hormone levels during a woman's lifetime, such as in puberty, pregnancy, and menopause, can make gums more

**Receding Gums: Causes, Treatment, Surgery, and Prevention**

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sensitive and more vulnerable to gum recession. Tobacco products. Tobacco users are more likely to have sticky plaque on their teeth that is difficult to remove and can cause gum recession. Grinding and clenching your teeth. Clenching or grinding your teeth can put too much force on the teeth, causing gums to recede. Crooked teeth or a misaligned bite. When teeth do not come together evenly, too much force can be placed on the gums and bone, allowing gums to recede. Body piercing of the lip or tongue. Jewelry can rub the gums and irritate them to the point that gum tissue is worn away.

**How Is Gum Recession Treated?** Mild gum recession may be able to be treated by your dentist by deep cleaning the affected area. During the deep cleaning -- also called tooth scaling and root planing -- plaque and tartar that has built up on the teeth and root surfaces below the gum line is carefully removed and the exposed root area is smoothed to make it more difficult for bacteria to attach itself. Antibiotics also may be given to get rid of any remaining harmful bacteria. If your gum recession cannot be treated with deep cleaning because of excess loss of bone and pockets that are too deep, gum surgery may be required to repair the damage caused by gum recession.

**What Type of Surgery Is Used to Treat Gum Recession?** The following surgical procedures are used to treat gum recession: **Open flap scaling and root planing:** During this procedure, the dentist or periodontist (gum doctor) folds back the affected gum tissue, removes the harmful bacteria from the pockets, and then snugly secures the gum tissue in place over the tooth root, thus eliminating the pockets or reducing their size. **Regeneration:** If the bone supporting your teeth has been destroyed as a result of gum recession, a procedure to regenerate lost bone and tissue may be recommended. As in pocket depth reduction, your dentist will fold back the gum tissue and remove the bacteria. A regenerative material, such as a membrane, graft tissue, or tissue-stimulating protein, will then be applied to encourage your body to naturally regenerate bone and tissue in that area. After the regenerative material is put in place, the gum tissue is secured over the root of the tooth or teeth. **Soft tissue graft:** There are several types of gum tissue graft procedures, but

**Receding Gums: Causes, Treatment, Surgery, and Prevention**

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the most commonly used one is called a connective tissue graft. In this procedure, a flap of skin is cut at the roof of your mouth (palate) and tissue from under the flap, called subepithelial connective tissue, is removed and then stitched to the gum tissue surrounding the exposed root. After the connective tissue -- the graft -- has been removed from under the flap, the flap is stitched back down. During another type of graft, called free gingival graft, tissue is taken directly from the roof of the mouth instead of under the skin.

Sometimes, if you have enough gum tissue surrounding the affected teeth, the dentist is able to graft gum from near the tooth and not remove tissue from the palate. This is called a pedicle graft. Your dentist can determine the best type of procedure to use on you based on your individual needs.

**How Can I Prevent Gum Recession?** The best way to prevent gum recession is to take good care of your mouth. Brush and floss your teeth every day and see your dentist or periodontist at least twice a year, or as recommended. If you have gum recession, your dentist may want to see you more often. Always use a soft-bristled toothbrush and ask your dentist to show you the proper way to brush your teeth. If a misaligned bite or teeth

grinding is the cause of gum recession, talk to your dentist about how to correct the problem. Other ways to prevent gum recession include:  
 Quit smoking if you smoke. Eat a well balanced and healthy diet. Monitor changes that may occur in your mouth.

By taking good care of your teeth, you can have a healthy smile forever.

#### 41. What may cause TMJ pain?

- a. Clenching and grinding
- b. Occlusal discrepancies from poor dentistry
- c. Airway space complications
- d. All of the above
- e. None of the above

#### TMJ (Temporomandibular Joint & Muscle Disorders) Overview

Temporomandibular joint and muscle disorders, commonly called "TMJ," are a group of conditions that cause pain and dysfunction in the jaw joint and muscles that control jaw movement.

Researchers generally agree that the conditions fall into three main categories: 1. Myofascial pain involves discomfort or pain in the muscles that control jaw function. 2. Internal derangement of the joint involves a displaced disc, dislocated jaw, or injury to the condyle. 3. Arthritis refers to a group of degenerative/inflammatory joint disorders that can affect the temporomandibular joint.

A person may have one or more of these conditions at the same time. Some estimates suggest that TMJ disorders affect over 10 million Americans. These conditions appear to be more common in women than men. Causes Trauma to the jaw or temporomandibular joint plays a role in some TMJ disorders, but in most cases, the exact cause of the condition is not clear. For many people, symptoms seem to start without obvious reason.

Because TMJ is more common in women than in men, scientists are exploring a possible link between female hormones and TMJ disorders. Symptoms A variety of symptoms may be linked to TMJ disorders. The most common symptom is pain in the chewing muscles and/or jaw joint. Other symptoms include:

- radiating pain in the face, jaw, or neck,
- jaw muscle stiffness,
- limited movement or locking of the jaw,
- painful clicking, popping or grating in the jaw joint when opening or closing the mouth,
- a change in the way the upper and lower teeth fit together.

Diagnosis There is no widely accepted, standard test now available to correctly diagnose TMJ disorders. Because the exact causes and symptoms are not clear, identifying these disorders can be difficult and confusing. Your doctor will note your symptoms, take a detailed medical history, and examine problem areas, including the head, neck, face, and jaw for tenderness, clicking, popping, or difficulty with movement. The doctor might also suggest imaging studies such as an x-ray.

You may want to ask your doctor about other causes of pain. Facial pain can be a symptom of many conditions, such as sinus or ear infections, various types of headaches, and facial neuralgias (nerve-related facial pain). Ruling out these problems first helps in identifying

TMJ disorders. Treatment Because more studies are needed on the safety and effectiveness of most treatments for jaw joint and muscle disorders, experts strongly recommend using the most conservative, reversible treatments possible. Conservative treatments do not invade the tissues of the face, jaw, or joint, or involve surgery. Reversible treatments do not cause permanent changes in the structure or position of the jaw or teeth. Even when TMJ disorders have become persistent, most patients still do not need aggressive types of treatment.

#### Conservative Treatments

Because the most common jaw joint and muscle problems are temporary and do not get worse, simple treatment may be all that is necessary to relieve discomfort. Short term use of over-the-counter pain medicines or nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen; the use of a stabilization splint, or bite guard, that fits over upper or lower teeth may provide relief. If a stabilization splint is recommended, it should be used only for a short time and should not cause permanent changes in bite. Studies of their effectiveness in providing pain relief have been inconclusive.

#### Irreversible Treatments

Surgical treatments are controversial, often irreversible, and should be avoided where possible. There have been no long-term clinical trials to study the safety and effectiveness of surgical treatments for TMJ disorders. Additionally, surgical replacement of jaw joints with artificial implants may cause severe pain and permanent jaw damage. Some of these devices may fail to function properly or may break apart in the jaw over time. Helpful Tips Self-care practices that may help ease symptoms of TMJ: • eating soft foods, • applying an ice pack, • avoiding extreme jaw movements like wide yawning, loud singing, and gum chewing, • learning techniques to relax and reduce stress, • practicing gentle jaw stretching and relaxing exercises that may help increase jaw movement. Your health care provider or a physical therapist can recommend exercises if appropriate for your particular condition.

Additional Resources • Less is Often Best in Treating TMJ Disorders NIDCR's fact sheet gives an overview of TMJ disorders. • Less if Often Best in Treating TMJ Ad Slicks [PDF - 3 pages] Three camera-ready ads suitable for use in newsletters, magazines, and other publications. These ads provide information on how to order NIDCR's "TMJ Disorders" brochure. • MedlinePlus: Temporomandibular Joint Dysfunction

The NIH National Library of Medicine's collection of links to government, professional, and non-profit/voluntary organizations with information on TMJ disorders.

- The TMJ Association
- The TMJ Association is a nonprofit, patient advocacy organization whose mission is to improve the quality of health care and the lives of everyone affected by temporomandibular disorders.

## TMJ DISORDERS

Temporomandibular joint and muscle disorders, commonly called "TMJ," are a group of conditions that cause pain and dysfunction in the jaw joint and the muscles that control jaw movement. We don't know for certain how many people have TMJ disorders, but some

estimates suggest that over 10 million Americans are affected. The condition appears to be more common in women than men.

For most people, pain in the area of the jaw joint or muscles does not signal a serious problem. Generally, discomfort from these conditions is occasional and temporary, often occurring in cycles. The pain eventually goes away with little or no treatment. Some people, however, develop significant, long-term symptoms.

If you have questions about TMJ disorders, you are not alone. Researchers, too, are looking for answers to what causes these conditions and what the best treatments are. Until we have scientific evidence for safe and effective treatments, it's important to avoid, when possible, procedures that can cause permanent changes in your bite or jaw. This booklet provides information you should know if you have been told by a dentist or physician that you have a TMJ disorder.

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### WHAT IS THE TEMPOROMANDIBULAR JOINT?

The temporomandibular joint connects the lower jaw, called the mandible, to the bone at the side of the head—the temporal bone. If you place your fingers just in front of your ears and open your mouth, you can feel the joints. Because these joints are flexible, the jaw can move smoothly up and down and side to side, enabling us to talk, chew and yawn. Muscles attached to and surrounding the jaw joint control its position and movement.

When we open our mouths, the rounded ends of the lower jaw, called condyles, glide along the joint socket of the temporal bone. The condyles slide back to their original position when we close our mouths. To keep this motion smooth, a soft disc lies between the condyle and the temporal bone. This disc absorbs shocks to the jaw joint from chewing and other movements.

The temporomandibular joint is different from the body's other joints. The combination of hinge and sliding motions makes this joint among the most complicated in the body. Also, the tissues that make up the temporomandibular joint differ from other loadbearing joints, like the knee or hip. Because of its complex movement and unique makeup, the jaw joint and its controlling muscles can pose a tremendous challenge to both patients and health care providers when problems arise.

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#### OPEN

Temporal muscle covering temporal bone

Disc

Condyle

Masseter muscle

Mandible

#### CLOSED

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### WHAT ARE TMJ DISORDERS?

Disorders of the jaw joint and chewing muscles—and how people respond to them— vary widely. Researchers generally agree that the conditions fall into three main categories:

- 1 Myofascial pain involves discomfort or pain in the muscles that control jaw function.
- 2 Internal derangement of the joint involves a displaced disc, dislocated jaw, or injury to the condyle.

3 Arthritis refers to a group of degenerative/ inflammatory joint disorders that can affect the temporomandibular joint.

A person may have one or more of these conditions at the same time. Some people have other health problems that co-exist with TMJ disorders, such as chronic fatigue syndrome, sleep disturbances or fibromyalgia, a painful condition that affects muscles and other soft tissues throughout the body. These disorders share some common symptoms, which suggests that they may share similar underlying mechanisms of disease. However, it is not known whether they have a common cause.

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Rheumatic disease, such as arthritis, may also affect the temporomandibular joint as a secondary condition. Rheumatic diseases refer to a large group of disorders that cause pain, inflammation, and stiffness in the joints, muscles, and bone. Arthritis and some TMJ disorders involve inflammation of the tissues that line the joints. The exact relationship between these conditions is not known.

How jaw joint and muscle disorders progress is not clear. Symptoms worsen and ease over time, but what causes these changes is not known. Most people have relatively mild forms of the disorder. Their symptoms improve significantly, or disappear spontaneously, within weeks or months. For others, the condition causes long-term, persistent, and debilitating pain.

TMJ

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#### WHAT CAUSES TMJ DISORDERS?

Trauma to the jaw or temporomandibular joint plays a role in some TMJ disorders. But for most jaw joint and muscle problems, scientists don't know the causes. Because the condition is more common in women than in men, scientists are exploring a possible link between female hormones and TMJ disorders.

For many people, symptoms seem to start without obvious reason. Research does not support the popular belief that a bad bite or orthodontic braces can trigger TMJ disorders. There is no scientific proof that sounds— such as clicking—in the jaw joint lead to serious problems. In fact, jaw sounds are common in the general population. Jaw noises alone, without pain or limited jaw movement, do not indicate a TMJ disorder and do not warrant treatment.

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TMJ

#### WHAT ARE THE SIGNS AND SYMPTOMS?

A variety of symptoms may be linked to TMJ disorders. Pain, particularly in the chewing muscles and/or jaw joint, is the most common symptom. Other likely symptoms include: n radiating pain in the face, jaw, or neck, n jaw muscle stiffness, n limited movement or locking of the jaw, n painful clicking, popping or grating in the jaw joint when opening or closing the mouth, n a change in the way the upper and lower teeth fit together.

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#### HOW ARE TMJ DISORDERS DIAGNOSED?

There is no widely accepted, standard test now available to correctly diagnose TMJ disorders. Because the exact causes and symptoms are not clear, identifying these disorders can be difficult and confusing. Currently, health care providers note the patient's

description of symptoms, take a detailed medical and dental history, and examine problem areas, including the head, neck, face, and jaw. Imaging studies may also be recommended. You may want to consult your doctor to rule out other known causes of pain. Facial pain can be a symptom of many conditions, such as sinus or ear infections, various types of headaches, and facial neuralgias (nerve-related facial pain). Ruling out these problems first helps in identifying TMJ disorders.

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TMJ

#### HOW ARE TMJ DISORDERS TREATED?

Because more studies are needed on the safety and effectiveness of most treatments for jaw joint and muscle disorders, experts strongly recommend using the most conservative, reversible treatments possible. Conservative treatments do not invade the tissues of the face, jaw, or joint, or involve surgery. Reversible treatments do not cause permanent changes in the structure or position of the jaw or teeth. Even when TMJ disorders have become persistent, most patients still do not need aggressive types of treatment.

**Conservative Treatments** Because the most common jaw joint and muscle problems are temporary and do not get worse, simple treatment may be all that is necessary to relieve discomfort.

**Self-Care Practices** There are steps you can take that may be helpful in easing symptoms, such as:

- n eating soft foods, n applying ice packs, n avoiding extreme jaw movements (such as wide yawning, loud singing, and gum chewing), n learning techniques for relaxing and reducing stress,

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- n practicing gentle jaw stretching and relaxing exercises that may help increase jaw movement. Your health care provider or a physical therapist can recommend exercises if appropriate for your particular condition.

**Pain Medications** For many people with TMJ disorders, short-term use of over-the-counter pain medicines or nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, may provide temporary relief from jaw discomfort. When necessary, your dentist or physician can prescribe stronger pain or anti-inflammatory medications, muscle relaxants, or antidepressants to help ease symptoms.

**Stabilization Splints** Your physician or dentist may recommend an oral appliance, also called a stabilization splint or bite guard, which is a plastic guard that fits over the upper or lower teeth. Stabilization splints are the most widely used treatments for TMJ disorders. Studies of their effectiveness in providing pain relief, however, have been inconclusive. If a stabilization splint is recommended, it should be used only for a short time and should not cause permanent changes in the bite. If a splint causes or increases pain, or affects your bite, stop using it and see your health care provider.

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The conservative, reversible treatments described are useful for temporary relief of pain – they are not cures for TMJ disorders. If symptoms continue over time, come back often, or worsen, tell your doctor.

**Botox** Botox® (botulinum toxin type A) is a drug made from the same bacterium that causes food poisoning. Used in small doses, Botox injections can actually help alleviate

some health problems and have been approved by the Food and Drug Administration (FDA) for certain disorders. However, Botox is currently not approved by the FDA for use in TMJ disorders.

Results from recent clinical studies are inconclusive regarding the effectiveness of Botox for treatment of chronic TMJ disorders. Additional research is under way to learn how Botox specifically affects jaw muscles and their nerves. The findings will help determine if this drug may be useful in treating TMJ disorders.

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**Irreversible Treatments** Irreversible treatments that have not been proven to be effective – and may make the problem worse – include orthodontics to change the bite; crown and bridge work to balance the bite; grinding down teeth to bring the bite into balance, called “occlusal adjustment”; and repositioning splints, also called orthotics, which permanently alter the bite.

**Surgery** Other types of treatments, such as surgical procedures, invade the tissues. Surgical treatments are controversial, often irreversible, and should be avoided where possible. There have been no long-term clinical trials to study the safety and effectiveness of surgical treatments for TMJ disorders. Nor are there standards to identify people who would most likely benefit from surgery. Failure to respond to conservative treatments, for example, does not automatically mean that surgery is necessary. If surgery is recommended, be sure to have the doctor explain to you, in words you can understand, the reason for the treatment, the risks involved, and other types of treatment that may be available.

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TMJ

**Implants** Surgical replacement of jaw joints with artificial implants may cause severe pain and permanent jaw damage. Some of these devices may fail to function properly or may break apart in the jaw over time. If you have already had temporomandibular joint surgery, be very cautious about considering additional operations. Persons undergoing multiple surgeries on the jaw joint generally have a poor outlook for normal, pain-free joint function. Before undergoing any surgery on the jaw joint, it is extremely important to get other independent opinions and to fully understand the risks.

The U.S. Food and Drug Administration (FDA) monitors the safety and effectiveness of medical devices implanted in the body, including artificial jaw joint implants. Patients and their health care providers can report serious problems with TMJ implants to the FDA through MedWatch at [www.fda.gov/medwatch](http://www.fda.gov/medwatch) or telephone toll-free at 1-800-332-1088.

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**IF YOU THINK YOU HAVE A TMJ DISORDER...**

Remember that for most people, discomfort from TMJ disorders will eventually go away on its own. Simple self-care practices are often effective in easing symptoms. If treatment is needed, it should be based on a reasonable diagnosis, be conservative and reversible, and be customized to your special needs. Avoid treatments that can cause permanent changes in the bite or jaw. If irreversible treatments are recommended, be sure to get a reliable, independent second opinion.

Because there is no certified specialty for TMJ disorders in either dentistry or medicine, finding the right care can be difficult. Look for a health care provider who understands musculoskeletal disorders (affecting muscle, bone and joints) and who is trained in treating pain conditions. Pain clinics in hospitals and universities are often a good source of advice,

particularly when pain continues over time and interferes with daily life. Complex cases, often marked by prolonged, persistent and severe pain; jaw dysfunction; co-existing conditions; and diminished quality of life, likely require a team of experts from various fields, such as neurology, rheumatology, pain management and others, to diagnose and treat this condition.

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TMJ

#### RESEARCH

The National Institute of Dental and Craniofacial Research (NIDCR), one of the National Institutes of Health (NIH), leads the federal research effort on temporomandibular joint and muscle disorders. In a landmark study, NIDCR is tracking healthy people over time to identify risk factors that contribute to the development of these conditions. Preliminary results from this study have identified a series of clinical, psychological, sensory, genetic and nervous system factors that may increase the risk of having chronic TMJ disorders. These new findings expand our scientific understanding of the onset and natural course of TMJ disorders and may lead to new diagnostic and treatment approaches.

**Pain Studies** Because pain is the major symptom of these conditions, NIH scientists are conducting a wide range of studies to better understand the pain process, including: n u n derstanding the nature of facial pain in TMJ disorders and what it may hold in common with other pain conditions, such as headache and widespread muscle pain, n pinpointing factors that lead to chronic or persistent jaw joint and muscle pain, n exploring differences between men and women in how they respond to pain and to pain medications, n e xamining the effects of stressors, such as noise, cold and physical stress, on pain symptoms in patients with TMJ disorders to learn how lifestyle adjustments can decrease pain, 1155 n identifying medications, or combinations of medications and conservative treatments, that will provide effective chronic pain relief, n i nvestigating possible links between osteoarthritis and a history of orofacial pain.

**Replacement Parts** Research is also under way to grow human tissue in the laboratory to replace damaged cartilage in the jaw joint. Other studies are aimed at developing safer, more life-like materials to be used for repairing or replacing diseased temporomandibular joints, discs, and chewing muscles.

#### HOPE FOR THE FUTURE

The challenges posed by TMJ disorders span the research spectrum, from causes to diagnosis through treatment and prevention. Researchers throughout the health sciences are working together not only to gain a better understanding of the temporomandibular joint and muscle disease process, but also to improve quality of life for people affected by these disorders.

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#### 42. What statement is incorrect about tooth whitening?

- When performed in the dental office, tooth whitening is permanent as long as patients brush and floss daily.
- In-home tooth whitening with custom trays has no negative health consequences.
- Activated charcoal has been shown to effectively whiten teeth with no negative health consequences.
- All are correct.
- All are incorrect.

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The authors declare that they have no conflict of interest.

#### Abstract

**Background:** At-home bleaching is a technique characterized by the use of carbamide peroxide or hydrogen peroxide as a tooth whitening agent. However, no data exists regarding systemic safety of this technique. The aim of this study was to investigate the effect of at-home bleaching on serum redox homostasis.

**Methods:** Twenty-nine healthy volunteers who requested for tooth whitening participated in this study.

Specified bleaching trays were fabricated for the maxilla and mandible arches. Each participant was given two

syringes containing 9% hydrogen peroxide gel to use for 30 min/night for 14 consecutive nights. To evaluate

the redox status, the serum concentrations of malondialdehyde (MDA), total antioxidant capacity (TAC), and

prooxidant-antioxidant balance (PAB) were measured. Blood samples were obtained in the morning prior to

initiation of study and the morning after on expiration of the bleaching period. The collected data were analyzed

using the t-test with confidence interval of 95%.

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Results: Twenty-three subjects completed the study. The MDA, PAB, and TAC were increased significantly after the bleaching period (p-value = 0.001, 0.001 and 0.002, respectively).

Conclusion: At-home bleaching revealed the potential to disturb oxidant-antioxidant balance and induce oxidative stress.

Clinical relevance: unfavorable and potential side effects of at-home bleaching should be considered.

Key words: Dental bleaching; Hydrogen peroxide; Oxidative stress; Redox.

## Introduction

In recent decades, tooth whitening techniques have grown rapidly mainly due to the esthetic reasons resulting to improvement in the undesirable appearance of teeth. Two main bleaching techniques are in-office and at-home methods. At-home bleaching technique can be done either dentist-supervised or self administered using over the counter (OTC) devices (1).

The bleaching process is based on a chemical reaction between pigmented molecules within the organic matrix of tooth and the oxidizing agents. In the course of the bleaching procedure, long-chain pigmented molecules are oxidized and split into smaller lighter molecules as well as other molecules including carbon, water, and oxygen (2). Different oxidizing chemicals were frequently applied to the tooth bleaching procedures. The main effective bleaching agent is hydrogen peroxide ( $H_2O_2$ ) as other materials break down to release this one. For instance, whitening products containing 10% carbamide peroxide release 3.5% hydrogen (1, 2). In the dentist-supervised at-home bleaching technique, also regarded as a conservative method of tooth whitening, the oxidative agents are held in contact with the tooth surface over a certain period in each day. In this method, the duration of exposure to the oxidizing agent depends on the concentration and type of bleaching agent (3).

A number of concerns over the safety of bleaching procedure have been raised by several researchers (4-7).

Among the side effects reported for the tooth bleaching procedure, includes slight reduction of tooth enamel, a temporary increase in tooth hypersensitivity, along with irritation of the mouth soft tissue especially gingival tissue (8-11).

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In addition to the transient and local side effects of the bleaching agents, the long term and irreversible systemic effects of these agents including genotoxicity, cytotoxicity, and carcinogenicity have been investigated (12).

Several animal studies have focused on these side effects. Ito et al.(13-16) reported the presence of adenoma and duodenum carcinoma along with hyperplasia following ingestion of 0.1% and 0.4% (w/v) of the hydrogen peroxide solution for 8 weeks in rats. Dahl and Pallesen(17) presented acute mucosal ulcerations in the stomach of rats following ingestion of carbamide peroxide in a dose-dependent manner. Timblin et al. (18) applied the hydrogen peroxide on human tracheal epithelium cells and observed over-expression of proto-oncogen-c-jun protein. However, conflicting reports over the long term effects of bleaching agents was observed along with no published report regarding these effects on humans.

It should be noted that there is less clinical control in at-home bleaching using a night-guard in comparison to the in-office bleaching. Additionally, the clinical control is least with over the counter devices without any dentist-supervision. Entrance of the oxidant agents (which are highly reactive) to the body circulation regardless of its entrance mean, could damage various cells and organs(19). Although there exists an intricate balance between oxidants (from internal metabolisms or exogenous sources) and antioxidants within the body cells, excessive oxidants could perturb this balance and lead to the oxidative stress (20); the result of which is damage to various organic compounds including lipids, proteins, and DNA (19). Moreover, high concentrations of the oxidants in comparison to the antioxidants can contribute to the pathogenesis of various types of cancers, cardiovascular diseases, and neurodegenerative disorders (21-23).

Till date, there is no published clinical study that addresses the systemic effects of bleaching agents, making this study the first to consider the potential systemic adverse impacts of these components. The current study aimed to investigate if the oxidizing agents used in the dentist-supervised at-home bleaching technique could lead to redox perturbation and subsequent oxidative stress after the bleaching period.

## Materials and Methods

### Study population

Twenty-nine (19 females and 10 males) healthy volunteers who requested for tooth whitening and referred to the Department of Operative Dentistry at Mashhad Dental School participated in the study.

All participants

signed a detailed informed consent and facilitated approval of this study by the Ethical Board of Mashhad

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University of Medical Sciences.

Dry mouth, enzymatic disorders, respiratory or digestive tract disorders, allergy to vinyl, tooth decay, exposed

root surfaces, broken teeth, enamel erosions, grinding para-function, poor oral hygiene, along with smoking

were the exclusion criteria. In addition, volunteers with any systemic disorder

(hypertension, diabetes mellitus,

etc) as well as patients who had been using antioxidant supplements (vitamin C and vitamin E supplements)

were also excluded from this study. Participants were instructed against the use of fast foods or vitamin C/E

supplements during the study period.

Bleaching Procedure

An alginate impression (Bayer, Leverkusen, Germany) was obtained to produce a negative mold of both

maxillary and mandibular arches. Casts were poured subsequently with dental Stone powder (Tara, Iran). The

bleaching tray was fabricated with 0.035 inch vacuum formed sheets using a vacuum tray-forming machine

(Ultradent Products Inc., South Jordan, United States). The trays were trimmed to 2 mm of the gingival

margins. Two 3-ml syringes of 9% hydrogen peroxide gel (Perfecta® Bravo® Tooth Whitening Gel, Premier

Dental Products, United States) with close to natural pH were handed to each participant.

The patients were

instructed to place enough bleaching agent into the tray to cover the facial surfaces of the target teeth (which

were visible during smiling, laughing, and talking) and then put the loaded tray on their teeth. Obtaining

maximum benefit of the product and patient compliance, participants were asked to wear the bleaching trays for

30 min per night for 2 weeks (according to the manufacturer's instruction) every night after daily routine

flossing and brushing. After the loaded tray was seated, the patients were instructed to remove any trace of

excess bleaching material on gingival tissue to minimize gingival irritation and swallowing of the bleaching

agents. Subjects were cautioned to discontinue the use of bleaching agents in cases of tooth hypersensitivity or gingival problems and call the research team immediately.

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#### Blood sample

Five milliliters of venous blood sample was collected from all participants twice; once in the morning of the day of commencement of the bleaching procedure, and thereafter in the morning of the day after the last night of bleaching. After separating serum from the whole blood, samples were kept at  $-80^{\circ}\text{C}$  until the time of assaying.

#### Thiobarbituric Acid Reactive Substances (TBARS) Assay

The serum concentration of malondialdehyde (MDA), both as a product of lipid peroxidation and as a biomarker of oxidative stress, was measured using a commercial kit (Cayman Chemical, Michigan, United States, Item Number 10009055) based on an established method(24). The assay was based on the formation of a pink colored complex between MDA and thiobarbituric acid under high temperature ( $100^{\circ}\text{C}$ ) and acidic conditions. Absorbance of the MDA-TBA complex was read at 532 nm and the concentration of MDA in samples was determined using a standard curve provided using 0 to 5  $\mu\text{M}$  MDA.

#### PAB Assay

A method described by Alamdari et al. (25) was utilized to estimate the prooxidant-antioxidant balance. In summary, for plotting the standard curve, varying proportions of 250  $\mu\text{M}$  hydrogen peroxide (as a representative of pro-oxidants) with 3 mM uric acid in 10 mM NaOH (as a representative of antioxidants) were mixed, implying that the ultimate oxidant sample contained only hydrogen peroxide with a concentration of 250  $\mu\text{M}$ , while the ultimate antioxidant sample had only uric acid with a concentration of 3 mM. Thereafter, in each well of a 96-well plate, 10  $\mu\text{l}$  of each sample, standard or blank (distilled water) were mixed with 200  $\mu\text{l}$  of the working solution which had been prepared according to the protocol (Alamdari et al., 2008). After 12 min incubation in a dark place at  $37^{\circ}\text{C}$ , reactions were terminated through the addition of 100  $\mu\text{l}$  of 2 N

HCl to each well; thereafter, the absorbance of samples was read at 450 nm with a reference wavelength of 620 or 570 nm.

The values of the unknown samples were calculated according to the standard curve.

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#### Total Antioxidant Capacity (TAC) Assay

To estimate the serum total antioxidant capacity, a method described by Miller et al. (26) was used that is the basis of a commercial kit (Cayman Chemical, Michigan, United States, Item Number 709001). This assay was based on serum antioxidants capacity for preventing the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical to ABTS radical cation by Metmyoglobin. The bleaching effect of antioxidants on blue-green color of ABTS radical cation was found to be proportional to the total antioxidant capacity that was measured as a change in absorbance at 660 nm. The experimental results obtained were compared with that of Trolox, a tocopherol analogue, and were presented as millimolar Trolox equivalents. All other chemicals were purchased from Sigma-Aldrich Company (Sigma-Aldrich Inc., Taufkirchen, Germany).

#### Statistical Analysis

The data obtained before and after bleaching periods were collected. The normality of data distribution was evaluated using one-sample Kolmogorov-Smirnov test. The difference between two measurements was performed using paired-sample t-test or Wilcoxon Signed Rank test. In addition, due to sample size limitations, the effective variables' size was measured using Eta Squared test. SPSS version 11.5 software (SPSS, Chicago, IL, USA) was used with the confidence interval of 95%.

#### Results

Twenty-three patients (16 females and 7 males) completed the study while six of the registered participants did not continue the project to the end. Two participants had moved away and were unable to participate in serum sampling sessions; also, others did not apply the bleaching agents according to specified standard protocol. Subjects used bleaching gels for  $14 \pm 2.1$  nights. The age of the subjects was  $29.4 \pm 9.5$  years old (ranging from

18 to 53). None of the subjects who completed the study reported any signs of tooth hypersensitivity or gingival problems during the study.

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The MDA concentration (TBARS), PAB, and TAC were all normally distributed in our measurements.

According to paired-sample t-test, serum concentration of MDA after bleaching ( $4.77 \pm 1.87 \mu\text{M}$ ) was

significantly higher than that of pre-bleaching samples ( $3.14 \pm 1.00 \mu\text{M}$ ) with a p-value of 0.001 (Fig. 1). The effect size of TBARS test was observed to be 0.427.

For PAB assay, since the standard curve was plotted based on increasing concentrations of hydrogen peroxide,

the higher value of PAB indicated a higher degree of redox perturbation in favor of oxidants. In this study,

while the PAB value for pre-bleaching samples was  $77.84 \pm 14.40$ , a significantly higher value of  $115.36 \pm$

$26.18$  was noticed after bleaching (p-value < 0.001) (Fig. 2). The effect size of PAB test was found to be 0.820.

As shown in Fig. 3, serum TAC which is proportional to both total concentration and capacity of all

antioxidants presented in serum, revealed an increased amount after bleaching ( $1.22 \pm 0.07 \text{ mM}$ ) compared to

pre-bleaching value of  $1.17 \pm 0.06 \text{ mM}$ ; this increase was statistically significant (p-value = 0.002). The effect

size of TAC test was found to be 0.371.

#### Discussion

Findings of the current research indicated that at-home bleaching resulted to significant increase in serum level

of malondialdehyde. Despite the total antioxidant capacity being increased significantly, the prooxidant

antioxidant balance was significantly shifted in favor of pro-oxidants. Consequently, systemic oxidative stress

was observed at the end of the tooth bleaching period.

With the limited sample size of the current study, further analysis was performed along with determination of

the resulting size of each assay. Regarding the scale proposed by Cohen, the Eta Square value which equals and

exceeds 0.16 is assumed as large effect size (27). As a result, the consequent size of TBARS, TAC, and PAB

were altogether largely considered; implying that large proportion of variance in these assays is explained by

the at-home bleaching procedure.

Determination of the lipid peroxidation level reveals the amount of free-radicals since they can potentially

initiate the peroxidation reaction of polyunsaturated fatty acids. The product is unstable lipid peroxide which

decomposes to various compounds including malondialdehyde (MDA) (24). Lipid peroxidation refers to a

mechanism of cell injury and is an indicator of oxidative stress; the products of which play an important role in

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wound healing process by activating the arachidonic acid cascade in plants (28). These products also help in

killing bacteria and spores in tissue injuries (29). However, excessive lipid peroxidation leads to membrane

rupture and cytotoxicity (30). Most disease conditions and toxins have been found to increase lipid

peroxidation. The peroxidation products are responsible for further cytotoxicity (29).

Animal studies along with

a case report of a 16-month ingested hydrogen peroxide have shown the acute cytotoxicity of the bleaching

agents (12). Nevertheless, systemic oxidative stress in the dentist-supervised at-home bleaching procedure has

been observed as the MDA serum level was increased.

MDA concentration has been reported as the most practical and precise technique in detecting oxidative stress

(31). This marker has been used in investigating the role of oxidative stress in various diseases including

chronic obstructive pulmonary disease (COPD), coronary heart disease, and diabetes mellitus (32-34). Tug et al.

(34) reported that the level of MDA measured by means of TBARS was 3.46  $\mu\text{M}$  in healthy subjects, 5.22  $\mu\text{M}$

in post-exacerbation period of COPD, and 7.15  $\mu\text{M}$  during exacerbation period.

Furthermore, Khan and Baseer

(33) reported that the mean MDA concentration in healthy subjects and patients with the coronary heart disease

was 2.24  $\mu\text{M}$  and 3.46  $\mu\text{M}$ , respectively. They assumed this increase as an indication of oxidative stress. In the

present study, authors found comparable increase in the MDA concentration (3.14  $\mu\text{M}$  before and 4.77  $\mu\text{M}$  after

bleaching) which could be interpreted to mean as systemic oxidative stress due to exposure to the bleaching

agents.

Along with increasing lipid peroxidation reactions during bleaching, the total antioxidant capacity (TAC) was

also increased; this shows the body's reaction toward entrance of toxic agents in circulation. However, the magnitude of the TAC increase was not as large as the magnitude of the increase in serum MDA concentration; hence, redox perturbation observably occurred in favor of oxidants indicated by the PAB assay.

In addition to some biological reactions as endogenous sources of free radicals, external sources are xenobiotics, ionizing radiation, UV light, some drugs, cigarette, fast foods, etc (35-37). However, the human body contains effective protective mechanisms against harmful effects of free radicals. Superoxide dismutase, glutathione peroxidase, catalase, the thioredoxin system, albumin, ferritin, bilirubin, and uric acid are part of the natural antioxidant systems (38, 39). The nutrients such as  $\alpha$ -Tocopherol (vitamin E) and ascorbic acid (vitamin C) also play an important role in the antioxidant activity against oxidants (39). In this study, to eliminate interference of the oxidant as well as antioxidant compounds with effect of the bleaching agents, a number of

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exclusion factors such as the consumption of vitamin C/E supplements as antioxidants, cigarette smoking, drugs, and fast foods were defined as the oxidant sources.

The imbalance between oxidative species and the antioxidant components, in which there exists an exceedingly higher sum amount of endogenous and exogenous oxidants, leads to the oxidative stress (19). In the present study, oxidative stress was observed after completion of bleaching period (2 weeks period). Although no side effects on short term oxidative stress could be detected in this study, according to the observed redox perturbation, safety of long term bleaching in cases of long term bleaching (that is, tetracycline discolorations) should be taken into consideration. Cerebral infarction, cardiac ischemia due to gas embolism, gastrointestinal emphysema, and lung edema has been reported in cases with hydrogen peroxide ingestion (40-42). Moreover, gastrointestinal malignancy, pathological alterations in pre-malignant lesions, and morphological changes in oral tissues due to local contact of whitening agents has been reported in animal and human studies (12). Nonetheless, there is a gap between the animal or in vitro studies and human studies as no clinical trial study is present to investigate the possible systemic effects of the bleaching agents.

Although International Agency on Research on Cancer attributes no carcinogenicity risk to the professional use of the bleaching agents (43), a recent review suggests that long term use of hydrogen peroxide with high concentration could promote oral mucosa injury, genotoxicity, and carcinogenicity (12). The observed findings of this study supported the later viewpoint as entrance of the oxidizing agents to blood circulation during the dentist-supervised at-home bleaching pertains to two possible mechanisms: swallowing small amounts of the hydrogen peroxide during application with night-guard and subsequent absorption through gastrointestinal tract; or local absorption through gingiva. Each pathway aggravates concerns over contact of agents with oral mucosa or GI lining and the possibility of genotoxicity, and carcinogenicity in cases with long term bleaching period (44). Moreover, free radical species react and oxidize cellular components, and further causes damage to lipids, DNA, and proteins (19). These damages could also subsequently result in a range of diseases that has been previously mentioned (21-23). None of the subjects who completed the study reported any signs of tooth hypersensitivity or gingival problems during the tooth whitening period. It may be related to natural pH and thixotropic nature of this at-home bleaching agent as described by the manufacturer to ensure it stays in contact with the teeth.

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However, tooth bleaching is considered an important way to improve people's social life and also at-home bleaching is the most conservative as well as the most effective method of bleaching. Hence, the authors are currently performing another clinical trial to investigate whether this oxidative stress resulting from the at-home bleaching could be diminished via shifting prooxidant-antioxidant balance in favor of the antioxidants through the use of the antioxidants (vitamin C supplements) during the procedure. One of the limitations of the current study was that changes in redox status were not evaluated after the bleaching period to determine the time needed to fully recover from the oxidative stress.

#### Conclusions

Based on the limitations and results of the present study, it could be concluded that oxidative stress is a

consequence of the dentist-supervised at-home bleaching technique. Although this stress is probably transient, probable side effects of the short term oxidative stress should be taken into consideration. Additionally, in cases with long term bleaching procedures or in situations with no dentist supervision as in over-the-counter tooth whitening products, the side effects may be significant hence the concern should be properly considered.

#### Clinical Significances

The redox imbalance due to oxidative stress with application of the tooth whitening agents may induce some probable harmful effects. Technical issues as well as careful education of patients on how to use the bleaching agents and trays should be seriously considered to minimize the possibility of oxidizing agents' leakage.

### Abstract

#### *BACKGROUND:*

Sales of charcoal dentifrices and powders have rapidly emerged into the Internet marketplace. The authors conducted a literature review to examine the efficacy and safety of charcoal and charcoal-based dentifrices.

#### *METHODS:*

The authors searched the MEDLINE and Scopus databases for clinical studies on the use of charcoal and charcoal-based dentifrices and laboratory investigations on the bioactivity or toxicity of charcoal and charcoal-based dentifrices, published through February 2017. The authors used a defined search strategy to identify randomized, controlled clinical trials with a follow-up duration of 3 months or longer. In addition, the authors selected the first 50 consecutive charcoal dentifrices from Google.com and Amazon.com for ascertainment of product assortment and advertising promotions.

#### *RESULTS:*

The authors' literature search identified 118 potentially eligible articles. Thirteen studies reported brushing the teeth with raw charcoal or soot; however, none of these studies met the inclusion criteria. Two studies offered nonspecific caries reductions, 3 studies reported deleterious outcomes (increased caries, enamel abrasion, nonquantified negative impact), and 1 study indicated only that brushing with raw charcoal had no adverse effects on oral hygiene. Seven other studies reported only on the use of charcoal for oral hygiene. Internet advertisements included unsubstantiated therapeutic claims-such as antibacterial, antifungal, antiviral, and oral detoxification, as well as potentially misleading product assertions. One-third of the charcoal dentifrices contained bentonite clay, and 1 contained betel leaves.

#### *CONCLUSIONS:*

The results of this literature review showed insufficient clinical and laboratory data to substantiate the safety and efficacy claims of charcoal and charcoal-based dentifrices. Larger-scale and well-designed studies are needed to establish conclusive evidence.

#### *PRACTICAL IMPLICATIONS:*

Dental clinicians should advise their patients to be cautious when using charcoal and charcoal-based dentifrices with unproven claims of efficacy and safety.

## A RESEARCH NOTE

## EFFECT OF ACTIVATED CHARCOAL ON WATER-SOLUBLE VITAMIN CONTENT OF APPLE JUICE

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## ABSTRACT

The effect of activated charcoals (0, 0.5, 1.0, 2.0 and 3.0 g/L of powdered and granular activated charcoals) on the content of several water-soluble vitamins in apple juice was studied. Apple juice samples with activated carbon added were mixed for 0 (control), 5, 10, 20. and 30 min, respectively. The content of water-soluble vitamins was analyzed by HPLC. Considerable reduction in ascorbic acid (vit C), niacin, pyridoxine mtamin B&, thiamine (Vitamin B,) and biotin concentrations was found while there was a dramatic improvement in the color and clearness of apple juice. The highest decrement in water-soluble vitamins was obtained at 3.0 gL powdered activated charcoal. Statistical analysis of the data showed highly significant diflerences (P C 0.05, P c 0.01) in the water soluble vitamins, color and clearness of the apple juice samples between the dosages of activated charcoals but no significant differences induced by the mixing periods.

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## INTRODUCTION

Vitamins can be classified in two main groups: water-soluble and fat-soluble vitamins. Among the B group of water-soluble vitamins, both thiamin (B,) and pyridoxine (Bd are important vitamins. They play different specific and vital functions in metabolism, and their lack produces specific diseases (Moreno and Salvado 2000). Vitamins are relatively unstable, affected by factors such as heat, light, air, other food components and food processing conditions (Machlin 1991 ; Ottaway 1993). Because of the critical role of vitamins in nutrition and their relative instability, qualitative and quantitative analyses are needed in food manufacturing. HPLC is the preferred technique for vitamin separation because of its high selectivity (de Leenheer ef al. 1985). Use of activated charcoal for color and patulin control in the production of apple juice and apple juice concentrate isn't a novel procedure in the world. However, there is no published report investigating changes in the concentration of water-soluble vitamins of apple juice from activated charcoal treatment. The objective of this study was to determine the water-soluble vitamin, color and clearness of apple juice when powdered and granular activated charcoals in different levels and stages of application were investigated and to help the apple juice manufacturing industry select an appropriate procedure.

## MATERIALS AND METHODS

Materials Apple juice and activated charcoals [Powdered (Carbopal Gn-A ultra), Granular (Granucol FA)] were used as the materials. The apples (Golden delicious) used for the production of apple juice were obtained from a well- established local factory (Denizli, Turkey). The granular activated charcoal was provided by Erbsloh Geisenheim GmbH & Co. KG, Geisenheim, Germany and the powdered activated charcoal by LURGI Aktivkohle GmbH, Frankfurt, Germany. The granular activated charcoal is soluble when directly stirred into the solution. Some properties of powdered and granular activated charcoals are given in Table 1.

Reagents Acetonitrile (HPLC grade) and K2HP04 (extra pure) were obtained from Merck (Darmstadt, Germany). Water used in all the experiments was doubly distilled and deionized. The vitamin standards (ascorbic acid, niacin, pyridoxine, thiamine and biotin) were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Stock and standard solutions of water-soluble vitamins

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were prepared in water. For preparing calibration curve, five different concentration levels of each standard were used. Correlation coefficients of ascorbic acid, niacin, pyridoxine, thiamin and biotin based on the concentration (pg/mL) versus peak area (mAU) were found to be 0.99, 0.98, 0.99, 0.97 and 0.98, respectively.

#### TABLE 1. PROPERTIES OF ACTIVATED CHARCOALS

##### Specifications

Activated Charcoal Powdered Activated Charcoal Granucol Fa

Moisture (%) < 10 5 pH value 2-5 6 Ash (56. dry matter) c5 Molasses factor 0.8 f 0.2 0.3

Methylene blue adsorption (MBA) 10 60 Total surface area (dg) 1350 1600 Water-soluble mawr (25C. %)

Production of Apple Juice The apples were cut into quarters with stainless steel knives, crushed (Beko, model BKK 1146, Istanbul, Turkey) and pressed by using a hydraulic press (Bucher-Guyer AG, Niederweningen, Switzerland) to obtain cloudy (unclarified) apple juice. The cloudy apple juice was heated in a tubular heat exchanger (Armfield, Model Fn4, Chicago, IL) at 80C for 3-5 min and cooled down to 45-50C in a container in circulating cooling water. Then, 1 mL L<sup>-1</sup> of pectolytic enzyme (Pectinex 100 - L, Nova Nordisk, Istanbul, Turkey) was added and the temperature was kept in the stated range during treatment (2 h). Following the pectolytic treatment, 500 mg L<sup>-1</sup> of gelatin (Type A, 75-100 bloom, Sigma- Aldrich Chemie GmbH, Deisenhofen, Germany) and 2500 mg L<sup>-1</sup> bentonite (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) were added. After resting for about 1.5-2 h, the apple juice was filtered through a Whatman filter paper (grade 40, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) with a 8 µm particle retention under vacuum. The filtered juice was then pasteurized in a plate heat exchanger (Gemak Ltd. \$ti, Ankara, Turkey) at 90C for 1 min.

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For each treatment, 1,000 mL of the produced apple juice (12.0 brix- regulated) was used. The activated charcoals were added into a glass beaker at 0, 0.5, 1.0, 2.0 and 3.0 g/L concentrations and followed by stirring. In each case, the activated charcoal was removed by filtering through filter paper (hterlab, Cat. No 47.05.001, istanbul, Turkey) after stirring each sample for 5, 10, 20, and 30 min. All the treatments (dosage levels and mixing periods) were replicated.

**Sample Preparation** The original pH of the juice samples was not modified. The juice samples were centrifuged for 10 min at  $14 \times 10^3$  g (Sigma, Bioblock Scientific 2-16). Prior to HPLC analysis, all samples were filtered using FP 30145 CA-S filters (Schleicher & Schuell, Darmstadt, Germany) with 0.45  $\mu$ m (7 bar max) pore size.

**Methods** The determination of water-soluble vitamins was carried out by using a Shimadzu VP series high pressure liquid chromatography apparatus (Shimadzu Corp., Kyoto, Japan), as suggested by Sigma-Aldrich Co. (SUPELCO 2000, Bellefonte, PA). The mobile phase employed was KH<sub>2</sub>PO<sub>4</sub>-acetonitrile (99/1) with a flow rate of 1.0 mL/min. For the analysis, a Discovery C18 150X4.6 mm column (Cat. No: 504953, a UV-VIS diode array detector (Shimadzu, model SPD-M10 Avp) set at 220 nm, a LC-IOAT-VP Shimadzu HPLC pump and a Software program (Shimadzu) were used. A typical chromatogram of the apple juice on a discovery C-18 (150X4.6 mm I.D.) column using KH<sub>2</sub>PO<sub>4</sub>-acetonitrile (99/1) as the mobile phase with a flow rate of 1.0 mL/min is illustrated in Fig. 1. Measurement of color and clearness of 12 brix-regulated apple juice compared with distilled water was carried out spectrophotometrically (Shimadzu, Model UV-1201V, Kyoto, Japan). To measure the color, the spectrophotometer was adjusted at 440 nm, whereas to measure clearness, the spectrophotometer was set at 620 nm (Kadalkal and Nas 2002). The soluble solid (brix) was determined by using a digital refractometer (RFM Model 340, Istanbul, Turkey) (AOAC 1980). The pH was measured with pH meter (WTW GmbH & Co., Model 537, Weilheim, Germany) (AOAC 1980). All samples were diluted to 11.2 °Bx at the time of analysis.

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**Recovery of Water-soluble Vitamins** Apple juice samples containing known amounts of ascorbic acid, niacin, pyridoxine, thiamine and biotin were spiked with the different levels (25, 50, 75, 100, 200  $\mu$ g/L) of the vitamins to determine recovery of each vitamin. The average percentage recoveries of ascorbic acid, niacin, pyridoxine, thiamine and biotin in apple juice were found to be 96.86%, 95.3%, 102.1%, 101.9%, and 95.8%, respectively, for levels added at five different concentrations. The levels of ascorbic acid, niacin, pyridoxine, thiamine and biotin in apple juice samples were corrected for the average percent recoveries.

**Statistical Analysis** Statistical analysis of the data was performed using SAS (SAS Institute, SAS 1985, Cary, NC). When analysis of variance (ANOVA) revealed a significant effect ( $P < 0.05$ ,  $P < 0.01$ ), data means were compared with the least significant difference (LSD) test.

#### RESULTS AND DISCUSSION

To the best of our knowledge, there is no published research on the effect of activated charcoal on the water-soluble vitamin content of apple juice. Statistical analysis of the data showed that there were significant differences ( $P < 0.05$ ) in the water-soluble vitamins of the apple juice samples between the dosages of activated charcoals. However, no significant differences were determined between the mixing periods. Data for the 5 min mixing periods were used for the evaluation of the results because, the least mixing period is most useful in view of time possession for the plants. The pH value of apple juice ranged from 3.85 to 3.82 and brix from 11.20 to 11.28. The effects of different doses of activated charcoals with 5 min mixing periods are shown in Table 2. The water-soluble vitamin

content of a control sample was decreased by the treatment of activated charcoal. Increasing amounts of activated charcoal decreased the water-soluble vitamin level in apple juice. However, it was observed that 5 min of treatment of the apple juice with activated charcoal was sufficient with respect to other treatments. Powdered activated charcoal was more effective in reducing the water-soluble vitamins than the granular activated charcoal. The highest losses of ascorbic acid, niacin, pyridoxine, thiamine and biotin in apple juice were found to be 16.5 %, 31.5 %, 48 %, 53.5 %, and 44.2 %, respectively, with the treatment of 3 g/L powdered activated charcoal when compared with the control sample. The lowest losses of ascorbic acid, niacin, pyridoxine, thiamine and biotin in apple juice were found to be 3.1 %, 4.0 %, 8.8%, 9.3%, and 4%, respectively, with the treatment of 0.5 g/L

TABLE 2. THE EFFECT OF DIFFERENT DOSES OF POWDERED AND GRANULAR ACTIVATED CHARCOALS ON ASCORBIC ACID, NIACIN, PYRIDOXINE, THIAMINE AND BIOTIN CONTENT (per 100 g) OF APPLE JUICE SAMPLES

Activated charcoal dose (g/L)	Ascorbic acid (%)	Niacin (%)	Pyridoxine (%)	Thiamine (%)	Biotin (%)
Control	79.6 a*	130 a*	54 a*	430 a*	520 a*
0.5	76.1 b	121 bc	45.3 b	370 b	460 b
1.0	73.3 c	114.6 c	40.4 c	320 c	410 c
2.0	70.0 d	98.0 d	68.5 d	270 d	330 d
3.0	63.3 e**	63.3 e*	31.5 e	200 e	290 e

0.5 76.1 b 121 bc 45.3 b 370 b 460 b 771 b 124.7 b 49.2 bc 390 b 500 b

n 5:

1.0 73.3 c 114.6 c 40.4 c 320 c 410 c 748 c 118 c 45.6 c 350 c 450 c

0.5 70.0 d 98.0 d 68.5 d 270 d 330 d 701 d 101, Sd 34 d 260 d 350 d 719 d 112.9 d 39.8 d 2

101, Sd 34 d 260 d 350 d 719 d 112.9 d 39.8 d 2

~ ~ ~ ~ ~ ' Results are the mean of 5 min mixing periods with two replicates \*, \*\*, Different letters in the same column are significantly different at P < 0.05, P < 0.01, respectively.

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activated charcoal when compared with the control sample. The losses of ascorbic acid, niacin, pyridoxine, thiamine and biotin were 13.295, 23.5%, 38.1%, 37.2%, and 36.5%, respectively, with the treatment of 3.0 g/L granular activated charcoal. In general, activated charcoal varying from 0.5 to 2.0 g/L is used in apple juice plants (A& et al. 1992; Kadakal and Nas 2002). The effects of different doses of activated charcoal with 5 min mixing periods on the color and clearness value of apple juice are shown in Table 3. Statistical analysis of the data showed that there were significant differences (P < 0.05) in the color and clearness of the apple juice samples between the dosages of activated charcoal. However, no significant differences were determined in the color and clearness of the apple juice between the mixing periods.

TABLE 3. THE EFFECT OF DIFFERENT DOSES OF POWDERED AND GRANULAR ACTIVATED CHARCOALS ON COLOR AND CLEARNESS OF APPLE JUICE SAMPLES

Activated charcoal dose (g/L)	Colour [440 nm]	Clearness [620 nm]	Colour [440 nm]	Clearness [620 nm]
Control	63.3 e*	97.6 e**	63.3 e*	91.6 e**
0.5	70.0 d	98.0 d	68.5 d	97.9 d
1.0	74.60 c	98.3 c	72.4 c	98.0 c
2.0	83.7 b	99.6 b	81.6 b	98.5 b
3.0	89.4 a	99.8 a	86.8 a	99.2 a

0.5 70.0 d 98.0 d 68.5 d 97.9 d 1.0 74.60 c 98.3 c 72.4 c 98.0 c

2.0 83.7 b 99.6 b 81.6 b 98.5 b

3.0 89.4 a 99.8 a 86.8 a 99.2 a

Transmittance \*, \*\*, Different letters in the same column are significantly different at  $P < 0.05$ ,  $P < 0.01$ , respectively. Results are the mean of 5 min mixing periods with two replicates

Transmittance value of the control sample at 440 nm was 63.3%. The transmittance values of the juice samples increased with the treatment of activated charcoals. Linear increment on the transmittance value of the juice samples was determined as the dosages of activated charcoals were increased. The best improvement in color of the juice samples were observed with the treatment by powdered activated charcoal of 5 min at the level of 3 g/L.

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#### CONCLUSION

(1) Use of activated charcoal for color and patulin control in the production of apple juice isn't a novel procedure in the world. Powdered activated charcoal and granular activated charcoals have decreasing effect on the water-soluble vitamin content of apple juice. If it is not necessary for color and patulin control in the production of apple juice, activated charcoal should not be used.

(2) The concentration of water-soluble vitamins in apple juice samples decreased with the increasing amounts of activated charcoal. Powdered activated charcoal was more effective than granular activated charcoal.

(3) There was a linear relationship between the concentration of activated charcoal and ascorbic acid, niacin, pyridoxine, thiamine and biotin losses of the apple juice but no relationship with the mixing period.

(4) There was a linear relationship between the concentration of activated charcoal and improvement of color and clearness of apple juice.

#### ACKNOWLEDGMENT

We thank Hasan Gurgen (Director of KONFRUT Apple Juice Plant) and Necati Ozdemir for their contribution to the financial support of this work.

43. Today's ink pigments for tattoos have been extensively researched and are controlled by the FDA?

- a. True
- b. False

FDA Advises Consumers, Tattoo Artists, and Retailers to Avoid Using or Selling Certain Tattoo Inks Contaminated with Microorganisms

This Safety Alert was updated on May 21. It was originally published on May 17.

Audience

Consumers who are considering a new tattoo Tattoo artists Retailers of tattoo inks

Product

The following tattoo inks have been voluntarily recalled because they are contaminated with microorganisms:

Scalpaink SC (<https://www.accessdata.fda.gov/scripts/ires/index.cfm?Product=171051>),

Scalpaink PA (<https://www.accessdata.fda.gov/scripts/ires/index.cfm?Product=171052>),

and Scalpaink AL (<https://www.accessdata.fda.gov/scripts/ires/index.cfm?Product=171053>) basic black tattoo inks manufactured by Scalp

Aesthetics (all lots distributed from 8/6/2018 through 10/1/2018) – Recall Completion Date 5/17/2019 Dynamic Color - Black tattoo ink manufactured by Dynamic Color Inc (lots

12024090 (<https://www.accessdata.fda.gov/scripts/ires/index.cfm?Product=171519>)

and 12026090

(<https://www.accessdata.fda.gov/scripts/ires/index.cfm?Product=171447>)) Solid Ink-

Diablo (<https://www.accessdata.fda.gov/scripts/ires/index.cfm?Product=171546>) (red)

tattoo ink manufactured by Color Art Inc. (dba Solid Ink) (dba Antone's Ink) (lot 10.19.18)

Purpose

The FDA is alerting consumers, tattoo artists, and retailers of the potential for serious injury from use of tattoo inks that are contaminated with bacteria. Tattoo inks

FDA Advises Consumers, Tattoo Artists, and Retailers to Avoid...

<https://www.fda.gov/cosmetics/cosmetics-recalls-alerts/fda-advi...>

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contaminated with microorganisms can cause infections and lead to serious health injuries when injected into the skin during a tattooing procedure, since there is an increased risk of infection any time the skin barrier is broken.

Commonly reported symptoms of tattoo-ink-associated infections include the appearance of rashes or lesions consisting of red papules in areas where the contaminated ink has been applied. Some tattoo infections can result in permanent scarring. Indications of an infection can be difficult to recognize as other conditions (e.g., allergic reactions) may initially have similar signs and symptoms, leading to misdiagnosis and ineffective treatments.

Summary of Problem and Scope

The FDA has become aware of contaminated tattoo inks through its FY2018-2019 inspections of distributors and manufacturers, routine surveys of marketed tattoo inks, and subsequent microbiological analysis of sampled tattoo inks. The FDA has identified 6 tattoo inks contaminated with bacteria harmful to human health and is working with the manufacturers/distributors to remove all contaminated product from the market. The tattoo inks were manufactured or distributed by 4 firms inspected under an ongoing

assignment. Tattoo inks were analyzed using methods described in the Bacteriological Analytical Manual Chapter 23: Microbiological Methods for Cosmetics ([/food/laboratory-methods-food/bam-methods-cosmetics](#)), which is the general method used to determine bacterial contamination of cosmetics.

#### Recommendations for Consumers

Ask the tattoo artist or studio about the tattoo inks they use and avoid the recalled tattoo inks listed above, due to risk of infection and injury.

#### Recommendations for Tattoo Artists, and Retailers

Avoid using or selling the recalled tattoo inks mentioned above, due to risk of infection and injury.

#### FDA Actions

The FDA will continue to work with manufacturers and retailers to remove contaminated products from the market.

#### Reporting Problems to the FDA

Consumers who have experienced symptoms of infection or an injury after

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<https://www.fda.gov/cosmetics/cosmetics-recalls-alerts/fda-adv...>

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administration of a tattoo should consult their healthcare professional and inform their tattoo artist. Consumers should also consider reporting their injury to MedWatch: FDA's Safety Information and Adverse Event Reporting Program ([/safety /medwatch-fda-safety-information-and-adverse-event-reporting-program](#)). The FDA encourages consumers with questions about product safety to submit an inquiry, or to visit [www.fda.gov/fcic](http://www.fda.gov/fcic) ([/food/resources-you-food/industry-and-consumer-assistancecfsan](#)) for additional information.

Why Doesn't the FDA Regulate Tattoo Ink? Are there serious adverse effects to injecting industrial paint under your skin? Nobody really knows. The inks used are not FDA-approved.

September 21, 2017

Given the popularity of tattoos, one would expect the physical effects to be well known. But in fact, the question has only recently been examined, most recently with studies that suggest that tattoo ink can leach into the lymph nodes, and that tattoos may reduce sweating. Reduced sweating impedes the body's ability to cool off, potentially presenting problems for anyone who is heavily tattooed and exercising in the heat. But sweating aside, are there long-term risks to tattoos? Nobody really knows.

Many tattoo inks are chemically similar or even identical to commercial pigments used in printers or even paint. Are there serious adverse effects to injecting industrial paint under your skin? Nobody really knows. The largest regulator of food and personal items, the FDA, has authority over pigments used in external-use cosmetics, such as lipstick. Artificial pigments must be approved by the FDA and tested to ensure that they contain approved ingredients, but colors derived from natural sources are not tested at all. In practice, due to limited resources and a belief that cosmetics pose little health risk, approved cosmetic pigments are mostly regulated directly by the cosmetic industry.

Most tattoo inks are de facto unregulated.

There's a loophole, however, large enough for a body suit. The FDA only exercises oversight over cosmetic pigments used externally. Internal use, i.e. permanently inserting pigments into the skin, is not regulated by the FDA at all. In a bizarre catch-22, since the pigments are not FDA approved for use in tattoos, and only FDA pigments are covered by the industry's testing scheme, most inks are de facto unregulated (The FDA will act if an obvious health problem is identified). Furthermore, FDA ingredient labeling requirements only apply to products sold directly to consumers. Ink is sold wholesale in bulk to shops, so not only are the inks not FDA approved, the ingredients are kept secret from users.

That leaves the regulation of tattoos to the states, where there is enormous variability in oversight. Many states, not all, have some regulations regarding the practice of tattooing, but there are few regulations regarding the contents or safety of the ink. Research regarding longterm effects of modern pigments or how pigments react when tattoos are removed is almost completely lacking.

As things currently stand, there is not yet evidence of long term harm to most tattoo recipients, nor does the sweating study provide evidence of risk. While there have been a few infections caused by unsterile ink, licensed artists are mostly conscientious and infection transmission through tattoos is uncommon. (The infection rate is higher in informal settings such as prisons, or friends' basements.) However, the rate of allergic or other poor reactions has been increasing.

Tattooing is an ancient practice, but the modern explosion of tattoo popularity and chemical pigments takes the health risks into unknown territory.

44. Organic toothpaste with bentonite clay has been shown to be the best toothpaste to use for overall health.

- a. True
- b. False

Charcoal toothpaste may do harm and not much good (Reuters Health) - Charcoal toothpaste may be having a moment as a go-to brightening and whitening tool, but some dentists say these products might actually damage tooth enamel and make cavities more likely. At a minimum, any claims charcoal toothpaste marketers make have no scientific evidence behind them, the authors of a paper in the British Dental Journal warn.

"The evidence highlighting any potential benefits of charcoal toothpaste over regular toothpaste is severely lacking," said Dr. Joseph Greenwall-Cohen of the University of Manchester Dental School in the UK, one of the coauthors.

"In general, I would encourage all people to stick to regular toothpaste over charcoal toothpaste," Greenwall-Cohen said.

A wide variety of charcoal toothpastes and tooth powders are available on store shelves, and packaging often claims that these products are "natural" or "eco-friendly" or have "antibacterial" or "antifungal" properties, the paper notes. This may persuade consumers they're buying something good for the environment that can also help prevent or treat gum disease or other oral health problems.

“There is simply no scientific proof that these products are capable of detoxifying your mouth, offer any increased antimicrobial activities (antibacterial, antifungal, antiviral), or can fortify/remineralize/strengthen tooth structure,” said Dr. John Brooks, a researcher at the University of Maryland School of Dentistry in Baltimore who wasn’t involved in the paper.

There’s an outside chance that charcoal toothpaste might lure some people with poor oral health habits to suddenly start flossing and brushing after every meal, and if this happens it could be considered one benefit of these products, Greenwall-Cohen and colleagues write. But the problem is that people with poor oral hygiene who try charcoal toothpaste may actually find it damages their tooth enamel or increases their risk of cavities, they warn. Not much research to date has tested the safety and effectiveness of charcoal toothpaste against alternatives in head-to-head clinical trials.

Some small studies looking at the effects of charcoal toothpaste have, however, found that it may be too abrasive to tooth enamel. Charcoal may erode the outer layer of enamel on teeth, exposing interior tissue and increasing the risk of tooth decay, some of these studies suggest.

Charcoal may also cause cancer, Brooks, who has done research on charcoal toothpaste, said by email.

“I have concerns about the chronic exposure of the oral mucosa (tissue) to charcoal as the federal government has classified charcoal as a carcinogen,” Brooks said. “Another potential health concern we uncovered was that one-third of the 50 brands of charcoal toothpaste we investigated included bentonite clay, a mineral that may contain crystalline silica, another recognized carcinogen by the federal government,” Brooks said.

When consumers do seek out specific toothpaste ingredients, fluoride is what matters most, dentists say. Plenty of research has found brushing with a toothpaste that contains fluoride can help prevent tooth decay and cavities.

Potential adverse effects on animal health and performance caused by the addition of mineral adsorbents to feeds to reduce mycotoxin exposure

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# The Author(s) 2019

Abstract

The contamination of feed with mycotoxins is a continuing feed quality and safety issue, leading to significant losses in livestock production and potential human health risks. Consequently, various methods have been developed to reduce the occurrence of mycotoxins in feed; however, feed supplementation with clay minerals or mineral adsorbents is the most prominent approach widely practiced by farmers and the feed industry. Due to a negatively charged and high surface area, pore volume, swelling ability, and high cation exchange capacity, mineral adsorbents including bentonite, zeolite, montmorillonite, and hydrated sodium calcium aluminosilicate can bind or adsorb mycotoxins to their interlayer spaces, external surface, and edges. Several studies have shown these substances to be partly or fully effective in counteracting toxic effects of mycotoxins in farm animals fed contaminated diets and thus are extensively used in livestock production to reduce the risk of myc

otoxin exposure. Nevertheless, a considerable number of studies have indicated that these agents may also cause undesirable effects in farm animals. The current work aims to review published reports regarding adverse effects that may arise in farm animals (with a focus on pig and poultry) and potential interaction with veterinary substances and nutrients in feeds, when mineral adsorbents are utilized as a technological feed additive. Furthermore, results of in vitro toxicity studies of both natural and modified mineral adsorbents on different cell lines are reported. Supplementation of mycotoxin-contaminated feed with mineral adsorbents must be carefully considered by farmers and feed industry.

Keywords Mycotoxins .Mineral adsorbents .Toxicity .Health effects .Feed safety

Abbreviations VTMS Vinyltrimethoxysilane HDTM Hexadecyltrimethyl BAC Benzalkonium chloride CMAB Cetyltrimethylammonium bromide OBAC Octadecyldimethyl benzyl ammonium chloride ACO Acetylcholine chloride HDA Hexadecylamine CHO Chinese hamster ovary HDTMA Hexadecyltrimethylammonium CBMN Cytokinesis block micronucleus cytome

HSCAS Hydrated sodium calcium aluminosilicates

#### Introduction

Feed is an integral part of the food chain, and it plays an important role in the growth, welfare, and productivity of farm animal as well as in the composition, safety, and quality of livestock products (milk, meat, and eggs) in the food supply chain (Guerre 2016; Makkar 2016). A complete or finished feed is manufactured from a mixture of raw materials of plant, animal, industrial, and pharmaceutical origin, and formulated to achieve a range of objectives in animals regarding health and performance (Ittiphalin et al. 2015; Guerre 2016). A recent global feed survey shows world compound feed production has reached 1.1 billion metric tons; China, USA, Brazil, Russia, India, Mexico, Spain, and Turkey represent the top eight countries responsible for 55% of total global feed

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production (Alltech 2019). Furthermore, commercial production of animal feed takes place in 144 countries and generates an annual turnover of more than US\$400 billion (IFIF 2018). This is expected to increase, as the industry is under pressure to increase the amount of safe and nutritious feed, to meet the global demand for livestock products (Makkar and Ankers 2014). However, one of the greatest challenges facing farmers and the feed industry is the occurrence of mycotoxins in feed ingredients (Li et al. 2014; Pinotti et al. 2016).

Mycotoxins in feed are a potential risk for animal performance, health, and the safety of foods of animal origin. More than 400 mycotoxins have been identified; however, only few have attracted scientific and regulatory interest due to the huge difficulties of collecting sufficient data in terms of frequency of occurrence and toxicity. The regulated mycotoxins in many parts of the world include aflatoxin B1 (AFB1), deoxynivalenol (DON), ochratoxin

A (OTA), zearalenone (ZEN), fumonisin B1 (FB1), and trichothecenes A– (T-2 and HT-2) (Zain 2011). These mycotoxins are produced by *Aspergillus*, *Fusarium*, and *Penicillium* species (Sweeney 1998) and their production is influenced by factors such as agronomic practice, pre- and post-harvest climate conditions (temperature, moisture level and carbon dioxide) (Pitt and Miller 2016; Gilbert et al. 2017). Hence, contamination can occur during crop growth, storage, and transportation (Mannaa and Kim 2017). A number of mycotoxins surveys have been carried out to investigate the worldwide occurrence of mycotoxins in feed materials such as wheat, maize, soybean meal, and dried distillers' grains (Monbaliu et al. 2010; Rodrigues and Naehrer 2012; Streit et al. 2013; Kosicki et al. 2016). The toxic effects of mycotoxin on animal health is termed mycotoxicosis, the degree of toxicity depends on the type of mycotoxins, level ingested, exposure time, breed, age, sex, health status of animal, and other stress factors (Zaki 2012; Khatoon 2016; Ostry et al. 2016; Dellafiora and Dall'Asta 2017). To counteract mycotoxicosis in farm animals, the European Commission (EC) regulation on additives for use in animal nutrition was amended and a new regulation (EC No 386/ 2009) in the category of technological feed additives defines a new functional group of feed additives as “substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action.” (EC 2009). Consequently, several research groups have examined the potential of mineral adsorbents to bind mycotoxins in vitro, and

their protective effects against mycotoxicosis in vivo. The topic has been extensively reviewed by Huwig et al. (2001), Döll and Dänicke (2004), Avanti et al. (2005), Kolosova and Stroka (2011), Di Gregorio et al. (2014), Zhu et al. (2016), Wielogórska et al. (2016), Peng et al. (2018), and Vila-Donat et al. (2018); a summary of recent studies (2014–2019) carried out on the efficacy of mineral adsorbents to alleviate mycotoxicosis in broiler chicken is shown in Table 1. Data

on mycotoxin adsorption by mineral adsorbents with other animal species during the time period are scarce in the scientific literature. Other studies have also shown that mineral adsorbents may not be effective and can induce deleterious effects in farm animals when supplemented with feeds (Dwyer et al. 1997; Watts et al. 2003; Döll and Dänicke 2004; Lemke et al. 2001; Khatoon et al. 2018). As these agents are naturally abundant, inexpensive, and can be chemically modified to further increase mycotoxin-adsorption capacity, leading to an extensive use in livestock feeding, this review aims to highlight adverse effects in farm animals caused by the supplementation of animal feeds with mineral adsorbents and consequences of their interaction with veterinary substances and micronutrients in feeds.

#### Composition and structure of clay minerals

Clays are fine graded (average particle size <0.002 mm) natural rock or soil material that exhibit plasticity when moistened or non-plastic and hard when dried (Andrade et al. 2011). Aluminosilicates are the largest and most important class of clay minerals, composed of silica, alumina, and significant amounts of alkaline and alkaline earth ions (Moreno-Maroto and Alonso-Azcárate 2018). Within this class, phyllosilicates and tectosilicates have received much scientific attention because they have a wide range of applications (Srinivasan 2011; Ghadiri et al. 2015).

The basic structure of phyllosilicates is based on tetrahedral sheets (T) composed of individual tetrahedrons which share three out of four oxygens, and octahedral sheets (O) composed of

individual octahedrons that share apical oxygen and hydroxyl anion groups with cations such as aluminum and magnesium. The stacking of both sheets determines the chemistry and crystallography of each phyllosilicate. The T-O ratio of layer structure is used to classify phyllosilicates into 1:1 (T-O) and 2:1 (T-O-T) (Wang et al. 2015). Examples of phyllosilicates include montmorillonite, illites, bentonite, and kaolinite. Tectosilicates have a three-dimensional framework structure where in all the four oxygen atoms of tetrahedra are shared with other tetrahedra (Alaniz et al. 2012); thus, the T-O ratio is 1:2. Examples of tectosilicates include zeolite, quartz, and feldspar. A summary of main clay minerals or mineral adsorbents currently used for sequestering mycotoxins is shown in Table 2. Although phyllosilicates and tectosilicates are composed of O and T sheets as predominant building blocks, the physicochemical properties of these minerals including charge, polarity, expandability, cation exchange capacity (CEC), pH, particle size, surface area, swelling ability, and adsorption capacity are dependent on structure, composition, and geographical origin (Deepthy and Balakrishnan 2005; Ito and Wagai 2017). In terms of natural abundance, a large percentage of mineral adsorbents are found mostly in twenty-three

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countries: Mexico, Germany, Armenia, Turkey, Italy, Uzbekistan, Azerbaijan, Kazakhstan, Ukraine, Turkmenistan, Bulgaria, USA, Czech Republic, South Africa, Moldova, Greece, Indonesia, Japan, Australia, Kyrgyzstan, Russia, Belarus, Tajikistan (EHC 2005).

Potential adverse effects of mineral adsorbents

Mineral adsorbents are added to animal feed as a non-nutritive additive, to prevent lump formation (anti-caking agent and coagulant), improve farm animal performance and bind or reduce mycotoxins (Kolossova and Stroka 2012). The mechanism of action for sequestering mycotoxins remains controversial, and six mechanisms have been proposed: selective chemisorption, electron donating, hydrogen bonding, furan ring bonding, ion interactions and coordination between exchange cations and the carbonyl groups. However, most researchers believe the process is physical adsorption by ion exchange reaction and electrostatic interaction (Thimm et al. 2001; Deng et al. 2010; Wang et al. 2018). Similar

mechanisms have also been proposed for the adsorption of nutritional content in animal diets including proteins (Ralla et al. 2010; Alam and Deng 2017), micronutrients (Schmidhalter et al. 1994; Barrientos-Velázquez et al. 2016), as well as veterinary drugs (Devreese et al. 2013). Furthermore, rate of adsorption is dependent on the origin and physicochemical properties of adsorbents and takes place predominantly in the acidic pH range (Deng et al. 2010).

Currently, bentonite, kaolinite, clinoptilolite, palygorskite, and montmorillonite are commercially available in natural and

Table 1 In vivo studies on the adsorption of mycotoxins in broiler chicken fed contaminated diet

Adsorbents Level (g/kg)

Mycotoxins (mg/kg)

Duration (days)

Main effect of the inclusion of a mineral adsorbent to the contaminated feed

References

Bentonite 10 AFB1 (0.02) ZEN (2) OTA (0.1)

- 42 No significant difference was observed in the overall performance of bentonite-treated birds  
Pappas et al. (2014)  
Montmorillonite 5 T-2 (4) HT-2 (0.66)
- 42 Significantly improved growth, serum biochemical parameters and reduced the level of toxins in tissues.  
Yang et al. (2014)  
Bentonite 7 AFB1 (0.02) 21 No significant differences were seen in terms of feed intake and biochemical parameters measured  
Dos Anjos et al. (2015)  
Diatomaceous earth 7.5 AFB1 (0.02) 21 Significantly decreased feed intake, body weight and serum concentration of glucose, albumin and protein  
Anjos et al. (2016)  
Calcium bentonite 2 AFB1 (200, 400, 600, and 1800)  
21 Treatment reduced the accumulation of AFB1 residues in the liver  
Fowler et al. (2015)  
Bentonite 3.7, 7.5 AFB1 (0.1, 0.2, 0.6) OTA (0.15, 0.3, 1)  
21 Both concentrations of bentonite ameliorated toxic effects of 0.1 and 0.2 mg/kg AFB1, but no significant effects on OTA-treated birds  
Bhatti et al. (2016)  
HSCAS 5 AFB1 (2) FB1 (10)  
37 HSCAS did not have any significant effect on reduced body weight and feed intake induced by mycotoxins  
Sobrane Filho et al. (2016)  
Bentonite 10 AFB1 (0.1) OTA (0.1)  
42 Significantly reduced OTA concentration in liver and breast muscle by 4-fold and completely removed AFB1 residues.  
Pappas et al. (2016)  
Bentonite 5 AFB1 (2) 21 Improved growth performance and increased liver and kidney weight.  
Shannon et al. (2017)  
Bentonite 5 AFB1 (0.1, 0.2, 0.6); OTA (0.15, 0.3, 1)  
42 Decreased 41% of AFB1 residues in the liver of broiler chicken  
Bhatti et al. (2017)  
HSCAS 3 AFB1 (0.04) 21 Significantly improved growth performance, digestibility and reduced AFB1 in liver and kidney.  
Liu et al. (2018)  
Bentonite 5, 10, 20 OTA (0.15, 0.3, 1) 42 No significant effect on total antibody, immunoglobulin titres and lymphoproliferative responses.  
Khatoon et al. (2018)  
Aluminosilicate 1 AFB1 (2, 4) 21 Increased body weight and feed efficiency as well as haematological parameters and serum proteins.  
Nazarizadeh and Pourreza (2019)  
Modified HSCAS 5 T-2 (6) 14 Prevented T-2 toxin-induced decreased body weight, feed intake, protein and total calcium and phosphorus  
Wei et al. (2019)

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modified forms. Modified adsorbents tend to have higher mycotoxin-sequestering capacity, due to alteration in charge and surface properties of natural adsorbents, using modifiers such as acids, alkalis, and organic cations—ACO, VTMS, HDTM, BAC, OBAC, and CMAB (Baglieri et al. 2013; Nones et al. 2016; Wang et al. 2018). Concerning the safety of these agents, published studies are either contradictory or lack some degree of accuracy in their assessments. Negative effects induced in farm animals due to interaction with veterinary substances and micronutrients in feed as well as *in vitro* and *in vivo* toxicity of natural and modified mineral adsorbents are discussed as follows.

#### Micronutrients

Micronutrients including iron, iodine, calcium, magnesium, selenium, zinc, and vitamins are essential minerals or elements required in minute quantities (less than 100 mg/kg per day) by animals for proper functioning of enzymes and hormones, to maintain growth and development (Smith et al. 2018). Clays are considered to be a source of minerals for animals due to the possession of an anionic framework with well-defined microstructures containing chemical elements mostly alkali metal ions and trace elements (Bhaskaran and Gupta 2006; Suzanne et al. 2017). Moreover, these minerals are exchangeable during ion-exchange process that is largely influenced by pH and temperature, leading to either bioavailability of minerals or deficiency in the gastrointestinal tract (ingesta) of animal feed supplemented with mineral adsorbents (Lukman et al. 2013). For instance, the hydrochloric acid in the stomach (low pH) and bile salts in the intestine (high pH) may change the physiochemical properties of mineral adsorbents thereby enhancing their ion-exchange capacity. This process may lead to the release of minerals from the surface of the adsorbents into the surrounding milieu, thus increasing the concentration of minerals (in addition to mineral content of feed) in the systemic circulation and subsequent accumulation in the body (Park et al. 2002; Mascolo et al. 2004; Mambai et al. 2010). Also, the ion-exchange process, particularly for adsorbents with a high CEC, may lead to adsorption of minerals and nutrients from feed, resulting in the deficiencies of micronutrients such as iron, potassium, and vitamins in farm animals (Ralla et al. 2010). Several publications have reported symptoms of vitamin A deficiency in chickens given feed supplemented with 0.5–3% bentonite (Briggs and Spivey 1999; Laughland and Phillips 2000; Hashemipour et al. 2010). Moreover, Erwin et al. (1998) demonstrated through an *in vitro* study that sodium bentonite has a strong affinity for pure carotene and can as well bind non-carotenoid pigments in feed (Erwin et al. 1998). With regard to trace elements, adverse effects observed in farm animals have been attributed to the imbalance between dietary concentration of trace elements in feed and the amount of trace elements in the mineral adsorbents (Thilsing et al. 2007; Grosicki and Rachubik 2010; Yang et al. 2017). Supplementation of feed with either 10 or 20 g/kg of palygorskite significantly decreased lead and copper accumulation in breast and thigh muscles of broiler chickens (Cheng et al. 2016). Correspondingly, inclusion of 0.5–2% zeolite and bentonite to chicken diet decreased serum levels of zinc, copper, and manganese, while aluminium concentration was significantly increased (Chung et al. 1990; Ivan et al. 1992; Utlu et al. 2007; Schwaller et al. 2016; Toprak et al. 2016). European Food Safety Authority Panel on Additives and Products used in Animal Feed (EFSA FEEDAP Panel) warned of a potential binding of manganese when bentonite is used at a dosage higher than 0.5% in feeds (EFSA FEEDAP Panel

2011a). Hooda et al. (2004) and Seim et al. (2013) also demonstrated different mechanisms by which mineral

Table 2 Summary of physicochemical properties of mineral adsorbents commonly used for adsorbing mycotoxins (Deepthy and Balakrishnan 2005; Lantenois et al. 2008; Pushcharovsky et al. 2016)

Adsorbent	Structure	CEC (cmol/kg)	Surface area (m <sup>2</sup> /g)	Mode of formation
Bentonite	2:1 Lattice	53–83	370–490	Alteration of volcanic ash in marine environment or silica bearing rocks such as granite and basalt.
Kaolinite	1:1 Lattice	3–15	5–20	Rock weathering or by hydrothermal process at high temperature or at low temperature by the alteration of primary minerals (such as feldspar).
Montmorillonite	2:1 Lattice	80–100	70–800	Weathering products in soils at moderately high temperature (200 °C)
Palygorskite	2:1 Lattice	4–40	300–600	Alteration of precursor minerals or by precipitation from rock solution.
Activated carbon	Pore	–	300–4000	Pyrolysis of different kinds of organic materials such as lignin, coconut shell, peat, hard and soft wood, lignite coal and carbonaceous materials.
Zeolite (Clinoptilolite)	1:2 Lattice	180–600	500–700	Rock interaction with aqueous solution or fluid in a wide variety of geochemical environments.

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adsorbents (bentonite and halloysite) can inhibit the absorption of dietary iron in vitro (Hooda et al. 2004; Seim et al. 2013). As the levels at which minerals occur in clays varies and dependent on geographical origin, it is essential to know the appropriate amount of clay and trace elements to be included in feed, to ensure animal welfare and productivity is not impaired by dietary mineral imbalances. Furthermore, a quality control system that includes adequate milling, cleaning, drying, and sieving as well as analysis of elemental composition, purity, and microbiological examination of final product must be established.

**Veterinary substances**  
Due to non-specific effects of mycotoxin binders, EFSA has recommended evaluation of oral veterinary drugs in feed supplemented with a mycotoxin binder, to prove its safety towards binding of medical substances (EFSA FEEDAP Panel 2011a, b). Interaction of mineral adsorbents with veterinary substances has been reported for antibiotics such as tilmicosin, tylosin, paromomycin, doxycycline, and coccidiostats such as

monensin and salinomycin. Goossens et al. (2012) studied the effects of bentonite on the oral bioavailability of doxycycline in pigs fed trichothecene-contaminated feed. The authors observed an increased plasma concentration of doxycycline administered as a single bolus in animals fed 100 µg/kg of T-2 and 1 mg/kg DON-contaminated diet when compared to control group. They suggested a complex interaction leading to increased oral bioavailability of antibiotics may occur when animals are given mycotoxin-contaminated diet, mycotoxin binders, and antibiotics concurrently (Goossens et al. 2012).

Efficacies of monensin and salinomycin to prevent coccidiosis in chickens were reduced in the presence of 0.5% sodium bentonite (Gray et al. 1998). Addition of bentonite to chicken diet has been shown to be incompatible with the use of

robenidine and is expected to reduce the effectiveness of other coccidiostats at levels higher than 0.5% (EFSA FEEDAP Panel 2011a, b). A bacteriostatic feed additive, tylosin, was unable to prevent airsacculitis and other clinical symptoms caused by *Mycoplasma gallisepticum* in broiler chickens fed a diet supplemented with 2% bentonite (Shryock et al. 1994). An in vitro model simulating intestinal barrier was

designed by Devreese et al. (2013) using porcine small intestinal epithelial cell line-J2 (IPEC-J2), to study efficacy and drug interaction testing of mycotoxin binders. The model was used to examine the passage of tylosin through the intestine in the presence of 1% bentonite. Bentonite interacted with tylosin and decreased its passage through IPEC-J2 (Devreese et al. 2013). Also, an *in vitro* study on adsorption of doxycycline by six different adsorbents (four montmorillonite based-clay, sepiolite and leonardite-based binder) showed that less than 25% of the initial concentration of doxycycline was detected after 4 h of incubation at 37°C, suggesting that 75% of doxycycline was adsorbed by mineral adsorbents (De Mil et al. 2015). Furthermore, the *in vitro* results were validated using two of the montmorillonite-based clays, to study the pharmacokinetics and oral bioavailability of doxycycline *in vivo*. Results showed that the two binders significantly lowered the area under the plasma concentration–time curve of doxycycline by less than 60% when compared with the control group. Similar *in vivo* result was reported by Osselaere et al. (2012); they observed a significant alteration in pharmacokinetic profiles and oral bioavailability of oxytetracycline and amoxicillin. Additionally, significant concentration of oxytetracycline was found in the kidneys of treated birds (Osselaere et al. 2012) compared to control group. Taken together, if a mycotoxin binder decreases or enhances the oral absorption of drugs, it may have a significant consequence on animal health, withdrawal time of the antibiotics and potentially public health in terms of exposure to antibiotic residues.

#### *In vitro* toxicity

The *in vitro* toxicity of mineral adsorbents has been widely studied. Due to the potential of mineral adsorbents to enter the body through different routes including inhalation, ingestion, and dermal penetration, cell lines such as keratinocytes, alveolar macrophages, erythrocytes, endothelial, hepatocytes, epithelial, and fibroblasts have been used to investigate toxic effects of mineral adsorbents (Elmore 2003; Maisanaba et al. 2015a). These cell lines represent major organs where adsorbent particles are localized and accumulated when humans and animals are exposed to clay particles via different routes (Michel et al. 2014; Connolly et al. 2019; Boim et al. 2019). Several *in vitro* toxicity studies of clay minerals have suggested an interaction or crosstalk between the surface of clay particles and cellular receptors (Verma et al. 2012; Michel et al. 2014). Furthermore, techniques such as fluorescence microscopy, transmission electron microscopy, time-lapse video microscopy, and histocytological staining have been utilized to show uptake of clay particles by macrophages and lymphocytes (Bowman et al. 2011; Kuhn et al. 2014; Connolly et al. 2019) as well as internalization by cell types such as keratinocytes and hepatocytes, through endocytosis and micropinocytosis pathways (Michel et al. 2014; Kuhn et al. 2014; Castro-Smirnov et al. 2017; Connolly et al. 2019). Also, accessory minerals such as quartz and metal oxides including TiO<sub>2</sub> and ZnO contained within clays have been suggested to be responsible for toxicity induced in different cell lines, with degree of toxicity dependent on size, shape, surface properties, and chemical composition of the adsorbent (Geh et al. 2006; Li et al. 2010). To elucidate mechanism of toxicity, different biomarkers such as lactate dehydrogenase leakage, reactive oxygen species generation, superoxide dismutase inhibition, and

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malondialdehyde release have been assayed (Li et al. 2010; Zhang et al. 2010; Baek et al. 2012; Lordan et al. 2010; Maisanaba et al. 2014). Furthermore, comet, Ames, and

CBMN assays were used to detect DNA damage and chromosomal loss (Li et al. 2010; Maisanaba et al. 2015a). Mineral adsorbents used for the assays are generally prepared by making up a suspension and measuring the absorbance to determine the concentration, followed by a multi-step ultrasonication at 40% vibration amplitude to disperse the suspension. Finally, the known stocks are serially diluted in cell culture medium and vortexed vigorously before being applied to cells. The culture medium without mineral adsorbent is used as the control. The majority of publications on the topic found mineral adsorbents to be toxic while a smaller number observed little or no effects. An investigation of short and long term toxicity of montmorillonite in human normal intestinal cells (INT-407) by Baek et al. (2012) revealed that montmorillonite (20–1000 µg/mL) can inhibit cell proliferation, induce oxidative stress and membrane damage between 24 and 72 h, with more remarkable cytotoxicity after long-term exposure (10 days) (Baek et al. 2012). Apoptosis and oxidative stress were also observed in human B lymphoblast cells exposed to 120 µg/mL and 240 µg/mL of natural bentonite and bentonite modified with 10–15% of H<sub>2</sub>SO<sub>4</sub>, within 24 h of exposure (Zhang et al. 2010). Furthermore, oxidative stress induced by modified bentonite was significantly higher than that of natural bentonite (Zhang et al. 2010). In terms of cell viability, CHO (ovary) and HepG2 (liver) cells exposed to montmorillonite (100–1000 µg/mL) exhibited a reduced viability and cytotoxic effects in a dose-dependent manner after 12–24 h of exposure (Li et al. 2010; Nones et al. 2015). Houtman et al. (2014) and Maisanaba et al. (2017) also observed reduced cell viability in Caco-2 (intestine) and HepG2 cell lines exposed to adsorbents modified with HDTM, ACO, and VTMS at 24 and 48 h of exposure (Houtman et al. 2014; Maisanaba et al. 2017). Other researchers (Verma et al. 2012; Maisanaba et al. 2015b) did not observe any reduction in viability of Caco-2, human umbilical vein endothelial (HUVEC), and A549 (human alveolar epithelial) cells exposed to mineral adsorbents. This may be due to the concentrations tested and type of adsorbents used. For instance, Maisanaba et al. (2015b) used a very low concentration range (0–8 µg/mL) of montmorillonite and both Verma et al. (2012) and Liu et al. (2012) used halloysite clays (aluminosilicates) (Verma et al. 2012; Maisanaba et al. 2015b). Regarding the genotoxicity of mineral adsorbents, EFSA FEEDAP Panel found algae interspersed bentonite to be nongenotoxic or mutagenic at the highest concentration tested, 250 µg/mL (EFSA FEEDAP Panel 2016). In a recently published opinion (EFSA FEEDAP Panel 2017), it was also reiterated that bentonite is non-genotoxic. Likewise, Geh et al. (2006), Li et al. (2010), and Maisanaba et al. (2014) did not observe any genotoxic effects when cells were exposed to natural or unmodified mineral adsorbents. Nevertheless, contrary results were observed in the case of modified adsorbents. Exposure of HepG2 cells to 15.6 µg/mL organo-modified montmorillonite significantly increased the frequency of micronuclei by 2.7-fold compared to control group using both comet and CBMN assays. Additionally, genes involved in DNA damage, metabolism, and oxidative stress were upregulated (Maisanaba et al. 2016). Similarly, HepG2 and Caco-2 cells exposed to 88 µg/mL and 141 µg/mL respectively of modified montmorillonite showed a significant increase in DNA damage after 48 h of exposure (Sharma et al. 2010; Maisanaba et al. 2013). In vitro toxicity testing plays an important role in the analysis of toxic effects of chemical substances and helps to identify potentially hazardous chemicals in the environment in a rapid and cost-effective way (Maisanaba et al. 2015b). In vitro toxicological studies of mineral adsorbents suggest adsorbents, especially modified ones, may induce dose-dependent

deleterious effects on various cell lines. With respect to the dosage used in the studies, there were challenges in describing the in vitro kinetics in the culture medium and actual kinetics when cells in the target tissue are exposed to the chemical substances. As in vitro models do not accurately determine the consequences of in vivo exposure because of differences in cell phenotype, immune systems, diverse protein reactions, intracellular signaling and fluidic environment (Baek et al. 2012), it is imperative to also evaluate toxicity of mineral adsorbents in animal models, taking into consideration their various applications in livestock nutrition.

#### In vivo toxicity

As stated earlier, several studies have shown that mineral adsorbents can alleviate negative effects induced by mycotoxins in farm animals (Table 1), other studies however, have reported undesirable effects in animal groups fed diets (with or without mycotoxins) supplemented with mineral adsorbents. Khatoon et al. (2018) observed a significant reduction in the total immunoglobulin, lymphoproliferative response and total antibody in birds fed OTA-contaminated diet (0.15–1.0 mg OTA/kg) and 0.5–2% of bentonite clay (Khatoon et al. 2018). Significant decrease in creatinine, uric acid, and cholesterol serum levels were also seen in broiler chickens fed AFB1 plus 0.25% and 0.5% clinoptilolite (Maciel et al. 2007). Three milligrams per kilogram of AFB1 plus sodic montmorillonite (0.25 and 0.5%) significantly protected against the toxic effects of AFB1 in pigs; however, 0.5% of sodic montmorillonite significantly reduced the level of serum phosphorus (Franciscato et al. 2006).

In layer birds, inclusion of 2% clinoptilolite to diet contaminated with 2.5 mg AFB1/kg significantly decreased egg weight and egg yolk index (Rizzi et al. 2003). Significant Mycotoxin Res (2020) 36:115–126120

decrease in triiodothyronine and thyroxine hormones were also observed in birds fed 2.5–5% of bentonite and 1 mg/kg of AFB1 (Eraslan et al. 2005). Addition of 4 g/kg of aluminosilicates to female weaned piglets diet contaminated with 8.6 mg DON/kg and 1.2 mg ZEN/kg significantly decreased feed intake and serum concentration of cholesterol. Furthermore, treated animals exhibited significant increase in the activities of aspartate transaminase and  $\gamma$  glutamyltransferase as well as serum concentration of albumin (Döll et al. 2005). The efficacy of low-pH montmorillonites modified with HDTMA and HDA to sequester ZEN was investigated in vivo using uterine weight of mice (Lemke et al. 2001). Supplementation of 0.5% modified montmorillonites with ZEN-contaminated feed did not only reduce the body weight of animals but also enhanced the toxicity of ZEN (increase in uterine weight). The authors concluded that alkylamine groups may promote the uptake of ZEN from a contaminated diet and result in an enhanced toxicity (Lemke et al. 2001). In all the studies outlined, negative effects of adsorbents were observed not only in animal groups fed mycotoxin-contaminated diet with mineral adsorbents but also in group fed mycotoxin-free diet plus mineral adsorbents. Many of the studies that reported protective actions of mineral adsorbents against toxic effects of mycotoxins in vivo focused mainly on zootechnical parameters such as feed intake and body weight gain without investigating the potential negative (unwanted) effects of mineral binders, as previously discussed by Döll and Dänicke (2004) (Döll and Dänicke 2004). The studies generally used three experimental groups: a negative control group (mycotoxin-free diet), positive control group (mycotoxin-contaminated diet), and treated group (mycotoxin-contaminated diet and mineral adsorbent), to demonstrate the efficacy of the mineral binders. However, a group

of animals were not included (mycotoxin-free diet with mineral adsorbent) to investigate nonspecific effects of mineral binders, which may occur independently of mycotoxin contamination.

Therefore, the safety or efficacy of such mineral binders has not been proven in a satisfactory manner. Other studies that investigated the effects of dietary mineral adsorbents (not as mycotoxin binder) have observed various negative effects. Pigs fed a diet supplemented with 2.5% and 5% montmorillonite experienced hepatic histological changes including swelling, vacuolar and vesicular degeneration. Furthermore, antioxidant capacity, glutathione peroxidase and average daily feed intake were severely affected compared to control group (Zhao et al. 2017). The authors concluded that inclusion of montmorillonite to a diet at a concentration above 1% may not be safe for starter pigs. Prvulović et al. (2008) also investigated the effects of dietary supplementation of 0.5% clinoptilolite on performance and biochemical parameters of pigs. They found a significant increase in body weight gain of pigs in the first 90 days; however, after 120 days, there was a significant decrease in growth rate as well as increased activity of aspartate aminotransferase in serum of clinoptilolite-treated group (Prvulović et al. 2008). In commercial layers, Berto et al. (2013) found that 0.5% inclusion of clinoptilolite to feed led to a decrease in animal performance and eggshell quality (Berto et al. 2013). Similarly, the addition of 1–3% of sodium bentonite decreased specific gravity, yolk color index, feed consumption, and egg production compared to birds fed control diet (Roland 1988; Hashemipour et al. 2010). The global occurrence of mycotoxins in feeding ingredients is of great concern as it is considered to be a major risk factor for animal performance and human health. One of the strategies for mitigating the occurrence of mycotoxins in feed is the inclusion of mineral adsorbents to feed. Several authors have proven these substances to be effective in alleviating mycotoxicosis in farm animals fed contaminated feeds. The FEEDAP Panel have also assessed the efficacy and safety of natural bentonite, they concluded that bentonite with following composition:  $\geq 70\%$  smectite (dioctahedral montmorillonite),  $< 10\%$  opal and feldspar, and  $< 4\%$  quartz and calcite is effective for binding AFB1 and safe for all animal species when used at a maximum level of 20,000 mg/kg in complete compound feed. Several of these products have been approved by EFSA and are available on the European market as either feed additives or digestibility enhancers. However, in other countries, there is no regulation on the use of these products, and they are listed as raw material catalogues such as bentonite, kaolinite, HSCAS, and palygorskite. Moreover, very limited information is available on their composition or physicochemical properties. Therefore, utilization of such adsorbents as feed additives must be carefully considered by farmers and animal nutrition companies. With regard to modified mineral adsorbents, very few researchers have investigated the safety of these products and both their efficacy and safety have not been assessed by the FEEDAP Panel. As it is essential to verify the potential of a mineral adsorbent to adsorb mycotoxins in vitro and in vivo, their safety and potential interaction with nutrients and veterinary substances in feeds must also be considered, using a complete experimental design: non-contaminated diet (negative control); mycotoxin-contaminated diet (positive control); non-contaminated diet with the mycotoxin binder and mycotoxin-contaminated diet with mycotoxin binder.

In summary, available data have shown that both natural and modified adsorbents or mycotoxin binders can induce cytotoxic effects including oxidative stress, reduction in cell viability, apoptosis, and DNA damage. They can bind essential micronutrients and vitamins in feed leading to reduced feed conversion, immunosuppression, and low productivity in livestock animals. Moreover, they can interact with veterinary drugs, which may cause a decline or an increase in the oral absorption of drugs, leading to a potential therapy failure and higher levels of antibiotic residues in foods of animal origin. Mineral adsorbents may also contain variable amounts of Mycotoxin Res (2020) 36:115–126 121

accessory minerals (quartz, nontronite, erionite), heavy metals (lead, copper, cadmium), dioxins, and trace elements, which can induce toxicity in livestock animals as well as alter serum mineral profile and activities of enzymes such as glutamate dehydrogenase, aminotransferase, creatinine, and glutathione peroxidase. Funding information This work has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement no. 722634.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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#### 45. Which statement is true?

- Dentists have been successful with communicating the toxicities of the dental materials they use in their patient's mouth.
- Mercury restorations have been shown to remain stable in the mouth and do not leak free mercury.
- Nitrous oxide has been proven to be harmless and can be used frequently without concern.
- Chlorhexidine has been shown to be cytotoxic to human cells.

Cytotoxicity evaluation of chlorhexidine gluconate on human fibroblasts, myoblasts, and osteoblasts James X. Liu, Jordan Werner, Thorsten Kirsch, Joseph D. Zuckerman, Mandeep S. Virk 

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**Abstract Introduction:** Chlorhexidine gluconate (CHX) is widely used as a preoperative surgical skin-preparation solution and intra-wound irrigation agent, with excellent efficacy against wide variety of bacteria. The cytotoxic effect of CHX on local proliferating cells following orthopaedic procedures is largely undescribed. Our aim was to investigate the in vitro effects of CHX on primary fibroblasts, myoblasts, and osteoblasts. **Methods:** Cells were exposed to CHX dilutions (0%, 0.002%, 0.02%, 0.2%, and 2%) for either a 1, 2, or 3-minute duration. Cell survival was measured using a cytotoxicity assay (Cell Counting Kit-8). Cell migration was measured using a scratch assay: a “scratch” was made in a cell monolayer following CHX exposure, and time to closure of the scratch was measured.

**Results:** All cells exposed to CHX dilutions of  $\geq 0.02\%$  for any exposure duration had cell survival rates of less than 6% relative to untreated controls ( $p < 0.001$ ). Cells exposed to CHX dilution of 0.002% all had significantly lower survival rates relative to control ( $p < 0.01$ ) with the exception of 1-minute exposure to fibroblasts, which showed 96.4% cell survival ( $p = 0.78$ ). Scratch defect closure was seen in  $< 24$  hours in all control conditions. However, cells exposed to CHX dilutions  $\geq 0.02\%$  had scratch defects that remained open indefinitely. **Conclusions:** The clinically used concentration of CHX (2%) permanently halts cell migration and significantly reduces survival of in vitro fibroblasts, myoblasts, and osteoblasts. Further in vivo studies are required to examine and optimize CHX safety and efficacy when applied near open incisions or intra-wound application. **Key words:**

chlorhexidine, cytotoxicity, osteoblasts, myoblasts, fibroblasts

**Introduction** Surgical site infections (SSIs) pose major challenges for orthopaedic surgeons. SSIs have led to prolonged hospital stays, increased readmission rates, and adds significant burden to the cost of healthcare. Patients who have experienced SSIs have substantially greater physical limitations and reduction in their health-related quality of life<sup>6-10</sup>. In response to these concerns, multiple antiseptic agents have been used to prevent perioperative bacterial contamination of the wound<sup>1-3</sup>. Chlorhexidine gluconate (CHX) is a widely used antiseptic agent and is present in a variety of preparations to prevent infection, including preoperative skin cleansing, surgical site preparation, intraoperative irrigation, CHX impregnated postoperative dressings, and hand antisepsis<sup>4</sup>. Strategies that have recently gained interest involves topically painting wound edges with 2% chlorhexidine gluconate during primary or revision shoulder replacement surgery, CHX application to the wound prior to postoperative dressing application, and intraoperative dilute CHX wound irrigation to minimize infection rates. Used as a surface disinfectant for its excellent efficacy against a wide variety of bacteria, CHX is applied to the skin and allowed to dry for 3 minutes as per standard protocol. When used as directed, the minimal bactericidal concentration of CHX was found to be 0.078%<sup>5</sup>. ChlorPrep [(Becton, Dickinson and Company, Franklin Lakes, NJ) 2% CHX and 70% isopropyl

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alcohol] has demonstrated higher effectiveness when used as a preoperative surgical site preparation compared to products that did not contain CHX 6. The practice of intra-wound CHX irrigation has been increasing, particularly in arthroplasty procedures, with its clinical use mirroring that of dilute betadine lavage following total joint arthroplasty<sup>5</sup>. Popular agents such as Irrisept (0.05% Chlorhexidine gluconate in sterile water, Irrimax Corporation, Innovation Technologies, Inc., Lawrenceville, GA), which is FDA-approved as an intra-wound irrigation delivery system<sup>7</sup>, are currently being used as a lavage following component implantation and prior to wound closure. However, there is a paucity of orthopaedic literature regarding the safety of CHX as an intra-wound irrigation agent and peri-incisional topical antiseptic. Prior studies have demonstrated cytotoxicity to native proliferating cells that are demonstrable in both clinical and cell-based studies<sup>4,8-11</sup>, while other studies support its clinical safety<sup>7,12</sup>. Prior studies have shown CHX solutions to be cytotoxic to human fibroblasts, osteoblasts, and lymphocytes in a time and dose dependent manner<sup>4,8,13,14</sup>, which may possibly delay wound healing or lead to increased rates of wound dehiscence<sup>15-20</sup>. Multiple in vitro studies with CHX has demonstrated its cytotoxicity to fibroblast cells<sup>18,20,21</sup>. While fibroblasts are a critical cell type in wound healing, myoblasts, and osteoblasts are crucial for skeletal muscle repair and bone healing, respectively<sup>22-24</sup>. The purpose of this study was to investigate the effect of CHX on not only primary fibroblasts, but also myoblast and osteoblast cell viability and migration using in vitro cell culture techniques. Our primary hypothesis is that the clinically used concentration of CHX diminishes 1) cellular viability and 2) cell migration of fibroblasts, myoblasts, and osteoblasts, as measured by the percent survival of cells via the Cell Counting Kit-8 cell survival assay and the validated scratch test<sup>25</sup>, respectively.

**Materials and Methods** This is a controlled in vitro laboratory study which was performed using three primary human cell types: fibroblasts, myoblasts, and osteoblasts. The effect of different CHX concentrations at either 1, 2, or 3-minute exposure durations on cell migration, measured via the scratch test, and cell survival, measured using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc. Rockville, MD), was systematically tested. The short exposure durations were selected to reflect the range of durations of CHX exposure that are clinically utilized for its antiseptic application. All experiments were performed in triplicate. Cell Culture Primary human fibroblasts (Lonza, Walkersville, MD, CC-2511) were cultured in Fibroblast Basal Medium (Lonza CC-3131) supplemented with human fibroblast growth factor-basic, insulin, gentamicin/ amphotericin-B, and 10% fetal bovine serum (Lonza CC-4134). Primary human myoblasts (DV Biologics, Yorba Linda, CA, AM002-F) were cultured in Muscle Cellutions Medium (DV Biologics M-GRO-001-500) supplemented with basal media supplement (DV Biologics M-GRO-0010-S) and 20 ng/ml of human fibroblast growth factor-basic (R&D systems, Minneapolis, MN, 233-FB-025). Primary human osteoblasts (Lonza CCC-2538) were cultured in Osteoblast Basal Medium (Lonza CC-3208) supplemented with 10% fetal bovine serum, ascorbic acid, and gentamicin/amphotericin-B (Lonza CC-4193). Cells were initially seeded in standard sterile 75-cm<sup>2</sup> tissue culture flasks (Corning Life Sciences, Tewksbury, Massachusetts) at a density of 10,000 cells/cm<sup>2</sup> and grown to 80% confluency at 37°C and 5% CO<sub>2</sub>. Cells were passaged in tissue culture flasks until passage 3, at which time the cells were seeded into 24-well plates (Corning Life Sciences, Corning, NY) at a density of 10,000 cells/cm<sup>2</sup> and cultured until 80-90% confluent. CHX solutions were made by diluting 4% chlorhexidine gluconate (Xttrium

Laboratories, Mount Prospect, IL) in sterile phosphate buffered saline (PBS, Gibco, Waltham, MA). Cell Survival Assay At 80% confluency, cells were washed with PBS, then exposed to either 0% (control), 0.002%, 0.02%, 0.2%, or 2% CHX for 1, 2, or 3 minutes, followed by 3X wash in PBS and reapplication of cell growth media. The concentrations of CHX were selected by using the concentration found in the most common surface preparation agent (ChloroPrep 2% Chlorhexidine gluconate), and serially lowering the concentration 3-fold on a logarithmic scale. Cells were returned to the tissue culture incubator at 37°C and incubated for 48 hours. After incubation, cells were washed again with PBS, and 10% Cell Counting Kit-8 (CCK) solution in cellular growth medium was applied to the cells. The CCK solution contains a highly soluble tetrazolium salt, which receives two electrons from viable cells, to generate an orange formazan dye, allowing colorimetric detection of cellular activity<sup>26</sup>. The cells were then incubated in the tissue culture incubator for 2 hours with the CCK solution, after which absorbance of cellular supernatants were

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measured at 450 nm according to the manufacturer's protocol. Absorbances of the experimental conditions were compared relative to control absorbance to calculate percent cell survival<sup>26</sup>. Scratch Test At 80% confluency, the cell monolayer was manually scraped in a straight line in the center of the well to create a "scratch" with a p200 pipette tip. The debris was removed by washing the cells with 1 ml of sterile PBS. After the scratch defect was made, cells were exposed to either 0% (control), 0.002%, 0.02%, 0.2%, or 2% CHX for 1, 2, or 3 minutes, followed by 3X wash in PBS and reapplication of cell growth media. To obtain the same field during image acquisition, reference points were made by marking the outer bottom of the dish with a fine permanent marker. The wells were placed under a phase-contrast microscope, leaving the reference mark just outside of the camera view. Images were captured of the cells before and immediately after the scratch defect was made, after which the cells were immediately returned to the tissue culture incubator at 37°C to avoid environmental changes. Subsequent images of the scratch defects were obtained at frequent intervals during the first 72 hours, then every 24 hours until day 14. Time until scratch defect closure, defined as the moment when the cells at the leading edge of the defect made contact with cells from the opposite side, were recorded for each experimental condition<sup>25</sup>.

Statistical Analysis The data was reported as mean  $\pm$  standard deviation. All cell survival results were compared relative to their respective controls using one-way analysis of variance (ANOVA). Statistical significance was set at  $p < 0.05$ . All statistics were performed using GraphPad Prism Version 7 (GraphPad Software, La Jolla, CA). Source Funding There was no external source of funding for this project. Results Cell Survival Assay The results of the cell survival assay are demonstrated in Figure 1. Fibroblasts (Fig 1A), myoblasts (Fig 1B), and osteoblasts (Fig 1C), exposed to CHX concentrations greater than or equal to 0.02% for any duration demonstrated significant reduction of cell survival to less than 6% relative to control ( $p < 0.001$ ). For exposure to 0.002% CHX, cell survival of myoblasts and osteoblasts was significantly reduced to all exposure times. However, cell survival rate of fibroblasts was significantly reduced following 2 and 3 minutes exposure ( $p < 0.01$ ) but not

to 1 minute exposure ( $p = 0.78$ ) of 0.002% CHX. Individual cell survival percentages and  $p$  values are listed in Table 1.

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Figure 1. A-C: Percent survival of cells 48 hours after exposure to different concentrations of chlorhexidine gluconate for 1, 2, and 3-minute durations. A) Fibroblast B) Myoblast C) Osteoblast

Scratch test The results of the scratch test are demonstrated in Figure 2. After 48 hours of incubation, fibroblasts, myoblasts, and osteoblasts demonstrate closure of the scratch defect under control conditions (exposure to PBS for 3 minutes). In 48 hours, all three cell types could not close their scratch defects after exposure to the clinically used concentration of 2% CHX for 3 minutes. Table 2 summarizes the observations in scratch defect closure at the 24-hour time point for fibroblasts, myoblasts, and osteoblasts after exposure to all CHX concentrations and exposure times. Any exposure to 0.02% CHX or greater resulted in open scratch defects (no scratch defect closure) in all cell types. Fibroblasts were able to close their scratch defect after exposure to 0.002% CHX for 1 and 2-minute exposure, but not for the 3-minute exposure. Myoblasts were unable to close the scratch defect after exposure to any CHX concentration for any duration. Osteoblasts were able to close their scratch defects after exposure to 0.002% CHX for 1-minute, but not for the 2-minute and 3-minute exposures.

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Figure 2. A-C. Scratch test results. At Time 0, scratch defects were initiated in monolayer cell cultures and exposed to either control conditions or the clinically used concentration of 2% CHX for 3 minutes. 48 hours later, photos were taken of the scratch defects to observe defect closure. White arrows demonstrate the width of scratch defect. Closure of the scratch defect is seen at 48 hours following exposure to control conditions for all cell types. Open scratch defects are seen at 48 hours following exposure to 2% CHX for 3 minutes. A) Fibroblasts, B) Myoblasts, C) Osteoblasts

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Table 1: Percent survival of cells 48 hours after exposure to different concentrations of chlorhexidine gluconate for 1, 2, and 3-minute durations. A) Fibroblast B) Myoblast C) Osteoblast.

Duration of CHX exposure

CHX Concentration

Percent cell survival  $\pm$  standard deviation

p-value, experimental condition vs. control

A - Fibroblast 1 minute Control  $100 \pm 15.3$  0.002% 96.4  $\pm$  14.3 0.78 0.02% 3.6  $\pm$  0.2 p < 0.001 0.2% 3.4  $\pm$  0.1 p < 0.001 2% 3.7  $\pm$  0.2 p < 0.001 2 minutes Control  $100 \pm 6.3$  0.002% 76.3  $\pm$  0.3 0.003 0.02% 3.2  $\pm$  0.1 p < 0.001 0.2% 3.2  $\pm$  0.2 p < 0.001 2% 3.2  $\pm$  0.2 p < 0.001 3 minutes Control  $100 \pm 6.0$  0.002% 27.9  $\pm$  10.3 p < 0.001 0.02% 1.8  $\pm$  0.01 p < 0.001 0.2% 1.9  $\pm$  0.1 p < 0.001 2% 1.8  $\pm$  0.1 p < 0.001 B - Myoblast 1 minute Control  $100 \pm 7.8$  0.002% 71.8  $\pm$  4.5 0.006 0.02% 1.4  $\pm$  0.03 p < 0.001 0.2% 1.7  $\pm$  0.1 p < 0.001 2% 1.5  $\pm$  0.1 p < 0.001 2 minutes Control  $100 \pm 9.5$  0.002% 61.3  $\pm$  6.7 0.004 0.02% 1.7  $\pm$  0.1 p < 0.001 0.2% 1.8  $\pm$  0.1 p < 0.001 2% 1.4  $\pm$  0.6 p < 0.001 3 minutes Control  $100 \pm 4.1$  0.002% 37.2  $\pm$  4.3 p < 0.001 0.02% 2.1  $\pm$  0.1 p < 0.001 0.2% 2.3  $\pm$  0.1 p < 0.001 2% 2.4  $\pm$  0.1 p < 0.001 C - Osteoblast 1 minute Control  $100 \pm 7.2$  0.002% 82.4  $\pm$  1.4 0.01 0.02% 4.1  $\pm$  0.03 p < 0.001 0.2% 3.5  $\pm$  0.3 p < 0.001 2% 3.8  $\pm$  0.1 p < 0.001 2 minutes Control  $100 \pm 8.6$  0.002% 25.5  $\pm$  2.9 p < 0.001 0.02% 4.5  $\pm$  0.1 p < 0.001 0.2% 3.5  $\pm$  0.1 p < 0.001 2% 3.2  $\pm$  0.1 p < 0.001 3 minutes Control  $100 \pm 7.2$  0.002% 24.5  $\pm$  4.0 p < 0.001 0.02% 5.6  $\pm$  0.1 p < 0.001 0.2% 5.6  $\pm$  0.4 p < 0.001 2% 5.8  $\pm$  0.1 p < 0.001

Table 2: Scratch test defect closure in fibroblasts, myoblasts, and osteoblasts 24 hours following CHX exposure for 1, 2, or 3 minute durations. "+" indicates closure. "-" indicates no closure.

	Fibroblast			Myoblast			Osteoblast		
	1 min	2 min	3 min	1 min	2 min	3 min	1 min	2 min	3 min
Control	+	+	+	+	+	+	+	+	+
0.002% CHX	+	+	+	+	+	+	-	-	-
0.02% CHX	-	-	-	-	-	-	-	-	-
0.2% CHX	-	-	-	-	-	-	-	-	-
2% CHX	-	-	-	-	-	-	-	-	-

Discussion The purpose of this study was to determine the effect of various concentrations of CHX on both cellular viability and cell migration of fibroblast, myoblast, and osteoblast cells in vitro. Cell migration, measured via the scratch assay in this study, is an essential process involved in tissue development, repair, and regeneration. We found that CHX has a significant cytotoxic effect on cell survival in vitro. These results are comparable to previous investigations that have evaluated the effect of CHX on fibroblast cells in vitro<sup>8,27-31</sup>. Alley et al. demonstrated that 0.12% CHX exposure for 3 minutes was significantly cytotoxic to ligament fibroblasts relative to control (p < 0.001)<sup>28</sup>. Wilken et al. demonstrated that 0.2% CHX exposure to human gingival fibroblasts resulted in immediate cell fixation onto tissue culture surfaces relative to control<sup>31</sup>. In this study we extended our investigation to include the myoblasts and osteoblasts in addition to fibroblasts. These three cell types play a significant role in wound healing, muscle repair, fracture healing, bony fusion, and osteointegration of uncemented arthroplasty implants. The clinically used 2%

concentration of CHX significantly reduced cell survival of all cell types, as well as permanently halted cell migration for all cell types, regardless of the exposure duration. The results of this data reinforce the need for further in vivo studies examining the safety and efficacy of CHX in the clinical environment. Furthermore, future in vivo studies are required with biocompatible agents that can be used clinically. Prior research has demonstrated the toxicity of CHX to human cells. Louis et al. 32 reported that exposure to 0.2% CHX disrupted the cell membrane of poly-morphonuclear leukocytes and caused fixation of their cytoplasmic contents. Goldschmidt et al. 30 reported that exposing fibroblasts to 0.004% CHX for 3-hours inhibited amino acid incorporation and even exposure for 10-minutes was able to prevent protein synthesis 4-hours later. In a study by Cline et al. 29, CHX was shown to affect fibroblast proliferation and impair cell adhesion. Chen et al<sup>21</sup>. exposed human gingival fibroblasts to 2% CHX and used the CCK assay to determine cell survival. In their study, cell viability was significantly reduced when exposed to 2% CHX for 3 minutes or longer. Similar to findings in this study, Chen et al demonstrated that cell viability was reduced in a time-dependent manner, with cells exposed to CHX for 10 minutes having cell survival rates significantly lower than the same cells exposed to CHX for only 3 minutes<sup>21</sup>. Flemingson et al.<sup>33</sup> exposed cultured human fibroblasts to different dilutions of commercially available CHX anti-plaque mouthwash products (1%, 2%, 5%, 10%, 20%, and 100%) for 1, 5, and 15 minutes. After exposure, the cells were rinsed twice with Minimum Eagle's

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Medium supplemented with 10% fetal bovine serum and cell survival was measured after 24-hours. They found that as CHX concentration increased there was a significant ( $p < 0.001$ ) difference in fibroblast proliferation – even at their lowest tested concentration (1%), exposure to CHX resulted in in vitro fibroblast survival of 51.7% compared to no exposure<sup>33</sup>. The dose-dependent cytotoxicity of CHX on fibroblasts, myoblasts, and osteoblasts is not a unique property of CHX; prior in vitro studies have demonstrated that dilute povidone-iodine and topical vancomycin powder, also used for prevention of surgical site infections, have similarly specific dose-dependent and time-dependent cytotoxicity profiles on fibroblasts, myoblasts, and osteoblasts, as measured via the scratch test and Cell Counting Kit-8 cell survival assay<sup>34,35</sup>. The bactericidal effect of CHX was not investigated in this study, but has been addressed in many previous studies<sup>5,15,20</sup>. Van Meurs et al. investigated the optimal dilution of various antiseptic solutions that resulted in minimal cytotoxicity against human fibroblasts and mesenchymal stromal cells while retaining a bacterial load reduction of  $> 99.9\%$ <sup>5</sup>. At 2-minute exposure durations, CHX was found to have no bactericidal effect at 0.2 g/L, and maximal bactericidal effect at 10 g/L, equivalent to 0.02% CHX and 1% CHX, respectively. The minimal bactericidal concentration (MBC) of CHX following 2-minute exposures was found to be 0.078%. Furthermore, they found CHX to have a cytotoxic effect on fibroblasts and mesenchymal cells at concentrations greater than 0.002%, leading to their conclusion that CHX was fully cytotoxic at concentrations well below the MBC. The study also concluded that, in an in vitro environment, the non-cytotoxic concentrations were less than the MBC for several other commonly used antiseptics, including polyhexanide, octenidine dihydrochloride, and povidone-iodine<sup>5</sup>. Our

study corroborates the cytotoxicity profile identified in this study, and also demonstrates similar cytotoxicity of CHX to osteoblasts and myoblasts cultured in vitro. In vivo clinical studies demonstrate mixed data regarding the safety and efficacy of CHX. When 1% CHX was accidentally used for irrigation during knee arthroscopy, the result was cartilage necrosis, non-specific inflammation and synovial fibrosis, persistent pain, swelling, and loss of knee range of motion<sup>9</sup>. Frisch et al. evaluated the effect of chlorhexidine irrigation on infection rates in 411 total joint arthroplasty patients. They were unable to discern a difference in infection rates or wound healing concerns between chlorhexidine irrigation and using dilute betadine for total hip arthroplasty and 0.9% saline for total knee arthroplasty<sup>7</sup>.

One of the limitations of this study is that an in vitro cell culture does not truly represent a surgical wound in vivo. Surgical wounds are usually vascularized, comprised of multiple cell types, utilize local and systemic inflammatory responses following tissue injury, and are under various mechanical forces that all affect wound healing. These conditions are not present in a monolayer culture and therefore necessitate in vivo studies to further investigate the effect of CHX. In vivo human progenitor cells are capable of regenerating, whereas in comparison, primary human cells grown in a monolayer tissue culture medium are limited in their regeneration capability. Studies have shown that in vivo human tissue generally has a higher tolerance for antiseptic solutions compared to in vitro tissue culture. It is currently unclear whether the cell death that occurs to the exposed cells of the wound bed will interfere with in vivo wound healing. However, controlled in vitro studies allows for better quantitative analysis on cell types without interference with in vivo factors. Despite these limitations, we have shown that even dilute CHX, at concentrations 100x below the clinically used concentration of 2% CHX, exert a significant cytotoxic effect on fibroblasts, myoblasts, and osteoblasts in cell cultures. The cytotoxicity of CHX on various cell types warrants further in vivo clinical studies that examine clinical results of direct CHX application adjacent to or inside open incisional wounds. Conclusion This study has shown that clinically used concentrations of CHX (2.0%) exerted a cytotoxic effect on osteoblasts, fibroblasts, and myoblasts in vitro. Decreased cell survival and the halting of cell migration were even seen at concentrations as low as 0.002% across all cell types, demonstrating the profound cytotoxic ability of CHX at concentrations far below that which is used clinically. While CHX is an effective topical antiseptic agent when used as directed prior to surgery, further clinical in vivo studies are required to characterize the effect on wound and tissue healing when CHX is used near open incisions, on postoperative dressings, or direct intra-wound application. With new agents such as CHX wound irrigations systems, CHX impregnated dressings, and the practice of peri-incisional CHX application becoming more common in clinical practice, it is important to evaluate the clinical effects of CHX application and determine the safest indications for use.

#### 46. What situation(s) can cause root sensitivity?

- a. Poorly placed tooth restoration.
- b. Aggressive subgingival flossing
- c. Heavy biting or chewing forces
- d. All are correct
- e. None is correct.

#### Tooth Sensitivity: Causes, Remedies & Treatment April 21, 2018

Tooth sensitivity is a common dental problem that involves discomfort or pain in teeth when encountering certain substances and temperatures. At least 40 million adults suffer from sensitive teeth in the United States, according to the Academy of General Dentistry. The pain is often sharp and sudden, but it is temporary. According to the Cleveland Clinic, the pain may shoot into the tooth's nerve endings. Fortunately, sensitive teeth can be treated and the condition can improve. Causes of tooth sensitivity There are no at-risk groups for tooth sensitivity. It can happen to anyone, according to Dr. Margaret Culotta-Norton, a dentist in Washington, D.C., and former president of the D.C. Dental Society. "The most common symptom ... is a sudden, sharp flash of pain when teeth are exposed to air, cold, sweet, acidic or hot foods," she told Live Science. Some people may experience tooth sensitivity from brushing or flossing their teeth.

In healthy teeth, enamel protects the underlying layer of dentin, which is softer than enamel. The tooth roots are protected by gums. But if the enamel is worn down or if the gum line has receded, then the dentin becomes exposed. "Cavities, cracked teeth, gum recession, enamel and root erosion all cause dentin to be exposed," Culotta-Norton said. "Dentin is connected to the nerve that triggers pain in sensitive teeth."

Dentin contains thousands of microscopic tubules, or channels, leading to the tooth's pulp, according to the Academy of General Dentistry. When exposed to the elements, these dentinal tubules allow heat, cold, acidic or even sticky substances to reach the nerves inside the tooth, causing pain.

According to the Cleveland Clinic, some factors that contribute to sensitive teeth may include:

- Brushing too hard or using a hard-bristled toothbrush. This can wear down enamel, causing dentin to become exposed, or encourage gum recession.
- Gum recession. This often happens in people suffering from periodontal disease, and it exposes the dentin.
- Gingivitis. Inflamed and sore gum tissue can result in exposure of the tooth's root.
- Cracked teeth. These can become filled with bacteria from plaque and cause inflammation in the pulp of the tooth. In more severe cases, it may lead to abscess and infection.
- Teeth grinding or clenching. This can wear down enamel.
- Plaque buildup.
- Long-term use of mouthwash. Some over-the-counter mouthwashes contain acids. If dentin is exposed dentin, the acids can make existing tooth sensitivity worse and also further damage the dentin layer. There are neutral fluoride mouthwashes available that might be a better option.
- Acidic foods. These can encourage enamel reduction.
- Dental procedures. Teeth may be sensitive after professional cleaning, root planing, crown replacement and other tooth restoration procedures. Usually the pain will disappear in four to six weeks. Tooth sensitivity after filling Some people may experience tooth sensitivity after having a cavity filled or a filling replaced, according to the Columbia University College of Dental Medicine.

The tooth decay that causes cavities irritates the tooth, and the filling procedure, while necessary, can lead to further sensitivity. Fortunately, tooth sensitivity after a filling should improve on its own within a few weeks. It may last longer, as much as a few months, but as long as the tooth sensitivity shows gradual improvement, there should be nothing to worry about. Persistent tooth sensitivity, however, may indicate that a root canal is needed. Sometimes after a filling, teeth become sensitive when biting down. This can be fixed with a simple bite adjustment. Additionally, the filling may be too high, according to Columbia University. In this case, the dentist could lower the filling. Composite fillings may cause tooth sensitivity when chewing. There is no pain when the teeth are clenched together, however. This tooth sensitivity is usually fixed by adjusting the bite or replacing the filling with another composite, according to Bear Peak Dental, a private practice in Boulder, Colorado. Tooth sensitivity after whitening treatments Teeth-whitening treatments — done either in a dentist's office or using an over-the-counter product — contain harsh chemicals that remove stains, but they can also remove the enamel, leading to tooth sensitivity. Several studies have investigated ways to lessen pain after in-office teeth-whitening treatments. A 2018 study in *Operative Dentistry* found that patients taking acetomeniphin/codeine before treatment did not reduce pain, so more dramatic measures are likely needed. A 2016 study in *Lasers in Medical Science* found that irradiating teeth with a lowlevel red laser with an infrared diode after a whitening treatment reduced pain levels significantly. A 2018 study published in *The Journal of the American Dental Association* found that applying a desensitizing gel before whitening significantly reduced tooth sensitivity after treatment. Another potential solution is changing the formula of whitening products. A 2017 study published in *Clinical Oral Investigations* found that reducing the acidity in bleaching gels resulted in significantly less pain with the same whitening results.

47. One study (A) has shown that milk can decrease root sensitivity. Another study (B) has shown that the application of propolis can decrease root sensitivity.

- a. Study A is true; Study B is false.
- b. Study A is false; Study B is true.
- c. Both Studies A and B are true.
- d. Both Studies A and B are false.

Dentin hypersensitivity: Recent trends in management Sanjay Miglani, Vivek Aggarwal, and Bhoomika Ahuja Department of Conservative Dentistry and Endodontics, Faculty of Dentistry, Jamia Millia Islamia, New Delhi - 110 025, India Address for correspondence: Dr. Sanjay Miglani, Department of Conservative dentistry and Endodontics, Faculty of Dentistry, Jamia Millia Islamia, New Delhi - 110 025, India. E-mail: moc.oohay@gimyajna Received 2010 Sep 13; Revised 2010 Sep 13; Accepted 2010 Sep 14. Copyright © Journal of Conservative Dentistry This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Abstract Dentinal hypersensitivity (DH) is a common clinical condition usually associated with exposed dentinal surfaces. It can affect patients of any age group and most commonly affects the canines and premolars of both the arches. This article concisely reviews the patho-physiology, mechanism and clinical management of the DH. Treatment of DH should start with an accurate diagnosis. Differential diagnosis should be made and all other probable causes should be excluded. An often neglected phase of clinical management of DH is the identification and treatment of the causative factors of DH. By removing the etiological factors, the condition can be even prevented from occurring or recurring. There are various treatment modalities available which can be used at home or may be professionally applied. The “at home” desensitizing agents include toothpastes, mouthwashes or chewing gums and they act by either occluding the dentinal tubules or blocking the neural transmission. This article also discusses the recent treatment options like bioglass, Portland cement, lasers and casein phosphopeptide. Keywords: Casein phosphopeptide - amorphous calcium phosphate, dentinal hypersensitivity, desensitizing agents, fluorides

INTRODUCTION Dentine sensitivity (DS) or dentinal hypersensitivity (DH) is one of the most commonly encountered clinical problems. It is clinically described as an exaggerated response to application of a stimulus to exposed dentine, regardless of its location.[1,2] The terms DS or DH have been used interchangeably to describe the same clinical condition. True hypersensitivity can develop due to pulpal inflammation and can present the clinical features of irreversible pulpitis, i.e., severe and persistent pain, as compared with typical short sharp pain of DH.[3] Majority of literature reviews dealing with this clinical condition have suggested the use of term DS and consider that the sharp pain is actually the normal pulpal response to the exposed dentine.[4,5] But it is well known that all exposed dentine are not sensitive and the term DH has been used over the decades by the clinicians.[6,7] Therefore, both the terminologies can be used to describe the clinical condition. The condition has been defined by an international workshop on DH as follows:[8] “Dentine hypersensitivity is characterized by short, sharp pain arising from exposed dentine in response to stimuli, typically thermal, evaporative, tactile, osmotic or chemical and which

cannot be ascribed to any other dental defect or pathology". Some authors have substituted the word "dentine" and added the site, such as cervical or root, resulting in various other terminologies (e.g., cervical sensitivity/hypersensitivity) to describe the same clinical condition.[9] PREVALENCE AND EPIDEMIOLOGY DH is a painful clinical condition with an incidence ranging from 4 to 74%.[10–14] The variations in the reports may be because of difference in populations and different methods of investigations. The methods employed are usually patient questionnaires or clinical examinations. Interestingly, the incidence of DH is much higher in patient questionnaires studies than in clinical studies which quote an incidence of mere 15%.[11,12]

A slightly higher incidence of DH is reported in females than in males. While DH can affect the patient of any age, most affected patients are in the age group of 20–50 years, with a peak between 30 and 40 years of age.[11] Regarding the type of teeth involved, canines and premolars of both the arches are the most affected teeth. Buccal aspect of cervical area is the commonly affected site.[15] ETIOPATHOGENESIS Anatomy of dentine pulp complex Dentine is covered and protected by hard tissues such as enamel or cementum. Dentin itself is a vital tissue, consisting of dentinal tubules, and is naturally sensitive because of extensions of odontoblasts and formation of dentine–pulp complex.[16] Although dentin and pulp are histologically different, their origin is embryologically from the same precursor, i.e., the ectomesenchyme.[16] Pulp is integrally connected to dentine, i.e., physiologic and/or pathologic reactions in one of the tissues will also affect the other. Dentin consists of small canal like spaces, dentinal tubules. These tubules occupied by odontoblastic processes.[16] The

odontoblastic processes may extend through the entire thickness of dentin from pulp to dentinoenamel junction. The odontoblastic processes are actually the extensions of odontoblasts, which are the major cells of pulp–dentin complex.[16] The odontoblastic processes are surrounded by dentinal fluid inside the tubules. The dentinal fluid forms around 22% of total volume of dentin.[16] It is an ultrafiltrate of blood from the pulp via dentinal tubules and forms a communication medium between the pulp (via the odontoblastic layer) and outer regions of the dentin. Pathogenesis It has been stated in the literature that DH develops in two phases: lesion localization and lesion initiation.[17]

Lesion localization occurs by loss of protective covering over the dentin, thereby exposing it to external environment. It includes loss of enamel via attrition, abrasion, erosion or abfraction. Another cause for lesion localization is gingival recession which can be due to toothbrush abrasion, pocket reduction surgery, tooth preparation for crown, excessive flossing or secondary to periodontal diseases.[18] As stated earlier, not all exposed dentine is sensitive. For DH to occur, the lesion localization has to be initiated. It occurs after the protective covering of smear layer is removed, leading to exposure and opening of dentinal tubules. MECHANISM Three major mechanisms of dentinal sensitivity have been proposed in the literature:

- Direct innervation theory • Odontoblast receptor • Fluid movement/hydrodynamic theory

According to direct innervation theory, nerve endings penetrate dentine and extend to the dentino-enamel junction.[19] Direct mechanical stimulation of these nerves will initiate an action potential. There are many shortcomings of this theory. There is lack of evidence that outer dentin, which is usually the most sensitive part, is innervated. Developmental studies have shown that the plexus of Rashkow and intratubular nerves do not establish themselves until the tooth has erupted; yet, newly erupted tooth is

sensitive.[16] Moreover, pain inducers such as bradykinin fail to induce pain when applied to dentine, and bathing dentine with local anesthetic solutions does not prevent pain, which does so when applied to skin.

Odontoblast receptor theory states that odontoblasts act as receptors by themselves and relay the signal to a nerve terminal.[20] But majority of studies have shown that odontoblasts are matrix forming cells and hence they are not considered to be excitable cells, and no synapses have been demonstrated between odontoblasts and nerve terminals.[21]

Brannstrom (1964) has proposed that dentinal pain is due to hydrodynamic mechanism, i.e., fluid force.[22] Scanning electron microscopic (SEM) analysis of “hypersensitive” dentin shows the presence of widely open dentinal tubules.[6] The presence of wide tubules in hypersensitive dentin is consistent with the hydrodynamic theory. This theory is based on the presence and movement of fluid inside the dentinal tubules. This centrifugal fluid movement, in turn, activates the nerve endings at the end of dentinal tubules or at the pulp–dentine complex.[21] This is similar to the activation of nerve fibers surrounding the hair by touching or applying pressure to the hair. The response of pulpal nerves, mainly A $\delta$  intradentinal afferent fibers, depends upon the pressure applied, i.e., intensity of stimuli.[21] It has been noted that stimuli which tend to move the fluid away from the pulp–dentine complex produce more pain. These stimuli include cooling, drying, evaporation and application of hypertonic chemical substances.[23] Approximately, 75% of patients with DH complain of pain with application of cold stimuli.[23] In spite of the fact that fluid movement inside the dentinal tubules produces pain, it should be noted that not all exposed dentine is sensitive. As stated before, the “hypersensitive” dentin has more widely open tubules and thin/under calcified smear layer as compared with “non-sensitive” dentine. The wider tubules increase the fluid movement and thus the pain response.[6,7]

#### CLINICAL MANAGEMENT OF DH

##### Diagnosis

As like any other clinical condition, an accurate diagnosis is important before starting the management of DH. DH has features which are similar to other conditions like caries, fractured or chipped enamel/dentine, pain due to reversible pulpitis, and post dental bleaching sensitivity.[15,24] Diagnosis of DH starts with a thorough clinical history and examination. The other causes of dental pain should be excluded before a definite diagnosis of DH is made. Some of these techniques include pain response upon the pressure of tapping teeth (to indicate pulpitis/periodontal involvement), pain on biting a stick (suggests fracture), use of transilluminating light or dyes (to diagnose fractures), and pain associated with recent restorations.[25] A simple clinical method of diagnosing DH includes a jet of air or using an exploratory probe on the exposed dentin, in a mesio-distal direction, examining all the teeth in the area in which the patient complains of pain.[26] The severity or degree of pain can be quantified either according to categorical scale (i.e., slight, moderate or severe pain) or using a visual analogue scale.[17]

##### Prevention of DH/removal of etiological factors

An often, neglected phase of clinical management of DH is the identification and treatment of the causative factors of DH. By removing the etiological factors, the condition can be even prevented from occurring or recurring. The etiological factors include faulty tooth brushing, poor oral hygiene [Figure 1], premature contacts, gingival recession because of periodontal therapy or physiological reasons, and exogenous/endogenous non-bacterial acids.[17]

Faulty tooth brushing includes hard brushes, excessive forces, excessive scrubbing at the cervical areas or even lack of brushing which causes plaque accumulation and gingival recession [Figures [Figures22 and and33].[27] The patient should be taught the correct method of tooth brushing with the help of a model. Highly abrasive tooth powder or pastes should be avoided.[17] Also, the patients should be instructed to avoid brushing for at least 2 hours after acidic drinks to prevent agonist effect of acidic erosion on tooth brush abrasion.

Erosive agents are also important agents in initiation and progression of DH [Figure 4]. They tend to remove the enamel or open up the dentinal tubules.[28,29] The erosive agents can be either exogenous dietary acids or endogenous acids. The exogenous dietary acids include carbonated drinks, citrus fruits, wines, yogurt, and professional hazards (workers in battery manufacturing, wine tasters).[28,29] A detailed dietary history should be taken. The quantity and frequency of the foods containing acids should be reduced. Patient should be advised to take something alkaline (milk) or at least neutral (water) after acidic drinks and to use a straw to sip the drink and avoid swishing it around the teeth. The endogenous acid comes from gastroesophageal reflux or regurgitation. It is also common in patients with eating disorders. The condition is characterized by generalized erosion of the palatal surfaces of maxillary anterior teeth.[30] Such a patient should be referred to the medical practitioner for expert management of the underlying disease. An occlusal splint can be fabricated to cover the affected areas, to prevent their contact with the acids.

#### CLASSIFICATION OF DESENSITIZING AGENTS I. Mode of administration

At home desensitizing agents

In-office treatment II. On the basis of mechanism of action

Nerve desensitization

- Potassium nitrate

Protein precipitation

- Gluteraldehyde • Silver nitrate • Zinc chloride • Strontium chloride hexahydrate

Plugging dentinal tubules

- Sodium fluoride • Stannous fluoride • Strontium chloride • Potassium oxalate • Calcium phosphate • Calcium carbonate • Bio active glasses ( $\text{SiO}_2\text{-P}_2\text{O}_5\text{-CaO-Na}_2\text{O}$ )

Dentine adhesive sealers

- Fluoride varnishes • Oxalic acid and resin • Glass ionomer cements • Composites • Dentin bonding agents

Lasers

- Neodymium:yttrium aluminum garnet (Nd-YAG) laser • GaAlAs (galium-aluminium-arsenide laser) • Erbium-YAG laser

Homeopathic medication

- Propolis At home desensitizing therapy Grossman[31] listed the requirements for an ideal dentine desensitizing agent as: rapidly acting with long-term effects, non-irritant to pulp, painless and easy to apply, and should not stain the tooth. Traditionally, the therapy for management of DH is primarily aimed at occluding the dentinal tubules or making coagulates inside the tubules.[17] Patients are often prescribed over-the-counter desensitizing agents. These "at home" desensitizing agents include toothpastes, mouthwashes and chewing gums.[17] Majority of the toothpastes contain potassium salts (potassium nitrate, potassium chloride or potassium citrate), sodium fluoride, strontium chloride, dibasic sodium citrate, formaldehyde, sodium monofluorophosphate and stannous

fluoride. Potassium salts act by diffusion along the dentinal tubules and decreasing the excitability of the intradental nerve fibers by blocking the axonic action.[32,33] Various clinical studies have shown the efficacy of potassium salts in controlling the DH.[34,35] It has been shown that toothpastes containing 5% potassium nitrate and 0.454% stannous significantly reduced the DH. Also, toothpastes containing potassium nitrate and fluorides have been shown to reduce postbleaching sensitivity.[36,37] The desensitizing toothpastes should be used with the help of a toothbrush with soft bristles. Patients should be advised to use minimal amount of water to prevent the dilution of the active agent. Along with the desensitizing toothpastes, mouthwashes and chewing gums containing potassium nitrate, sodium fluoride or potassium citrate are also recommended.[17] The results of “at-home” desensitizing therapy should be reviewed after every 3–4 weeks. If there is no relief in DH, “in-office” therapy should be initiated. In-office desensitizing agents Theoretically, the in-office desensitizing therapy should provide an immediate relief from the symptoms of DH. The in-office desensitizing agents can be classified as the materials which undergo a setting reaction (glass ionomer cement, composites) and which do not undergo a setting reaction (varnishes, oxalates). Fluorides

Traditionally, fluorides have been used as a caries preventive material which can help in remineralization of enamel/dentin.[38] Also, various clinical trials have shown that application of fluoride solution can decrease the DH.[39,40] Fluorides decrease the dentinal permeability by precipitation of calcium fluoride crystals inside the dentinal tubules.[17] These crystals are partially insoluble in saliva. SEM revealed granular precipitates in the peritubular dentin after application of fluorides.[41] Various fluoride formulations are used to treat DH. These include sodium fluoride, stannous fluoride, sodium monofluorophosphate, fluorosilicates and fluoride combined with iontophoresis.[17] Sodium fluoride has been used in dentifrices or may be professional applied in a concentration of 2%. The precipitates formed by sodium fluoride can be mechanically removed by the action of saliva or mechanical action. Therefore, an addition of acid formulation is recommended. The acidulated sodium fluoride can form precipitates deep inside the tubules. Also, some authors have recommended the use of iontophoresis along with sodium fluoride.[41,42] The electric current is supposed to increase the ion diffusion. A clinical study has shown that 0.4% stannous fluoride along with 0.717% of fluoride can provide an immediate affect after a 5 minute professional application.[43] Stannous fluoride acts in a similar fashion as that of sodium fluoride, i.e., formation of calcium fluoride precipitates inside tubules. Also, SEM studies have shown that stannous fluoride itself can form insoluble precipitates over the exposed dentine.[44] Fluorosilicates act by formation of precipitates of calcium phosphates from saliva. Ammonium hexafluorosilicate has been used as a desensitizing agent. It can present a continuous effect of dentinal tubule occlusion via precipitation of a mixture of calcium fluoride and fluoridated apatite.[45,46] If the precipitate is predominantly composed of fluoridated apatite, it can form stable crystals deposited deep inside the dentinal tubules.[45,46] These crystals are resistant to removal from the action of saliva, brushing or action of dietary substances. Oxalates Oxalates can reduce dentinal permeability and occlude dentinal tubules. Thirty percent potassium oxalate had shown a 98% reduction in dentinal permeability.[47] Also, topical application of 3% potassium oxalate reduced DH after periodontal therapy.[47] The oxalate reacts with the calcium ions of dentine and forms calcium oxalate crystals inside the dentinal tubules as well as on the dentinal surface. This

results in a better sealing as compared with an intact smear layer.[17] It has been shown that the effect of oxalates on DH diminishes over a period of time. This can be attributed to the removal of the calcium oxalate crystals by brushing or dietary acids. The condition can be improved by acid etching of the dentinal surface, thus increasing the penetration of calcium oxalate crystals deep into the dentinal tubules.[48] Many vegetables like rhubarb, spinach and mint contain oxalates. It has been shown that phytocomplexes obtained from these natural products can reduce the dentinal permeability. This can also be followed by covering the exposed surface with a dental adhesive.[49] Potassium oxalate can lead to gastric irritation. Therefore, it should not be used with a tray with prolonged placement. Varnishes are commonly used useful in-office measures to treat DH. Copal varnish can be applied to cover the exposed dentinal surface. But its effect is for short term and is not recommended for long term management of DH.[50] To improve its efficacy, removal of smear layer is advocated. Also, the varnishes can act as a vehicle for fluoride. The fluoride varnishes can be acidulated to increase the penetration of ions.[50]

**Adhesive materials** Resin-based dental adhesive systems can provide a more durable and long lasting dentine desensitizing effect. The adhesive resins can seal the dentinal tubules effectively by forming a hybrid layer.[17] Various clinical studies have demonstrated the effectiveness of adhesives in management of DH.[51–53] Traditionally, resin composites or dentin bonding agents are used as desensitizing agents. The conventional dentin bonding agents (DBA) removes the smear layer, etches the dentinal surface and forms deep dentinal resin tags inside the dentinal tubules. The combined dentin–resin layer (consisting of penetrating resinous tags) has been termed as hybrid layer. It effectively seals the dentinal tubules and prevents DH.[51–53] Newer bonding agents modify the smear layer and incorporate it in into the hybrid layer.[54] Recently, some dentin bonding agents have been introduced in the market with the sole purpose of treating DH. Gluma Desensitizer (Heraeus Kulzer, Hanau, Germany) contains hydroxyethyl methacrylate (HEMA), benzalkonium chloride, gluteraldehyde and fluoride. Gluteraldehyde causes coagulation of the proteins inside the dentinal tubules.[54] It reacts with the serum albumin in the dentinal fluid, causing its precipitation. HEMA forms deep resinous tags and occludes the dentinal tubules.[54] Gluma has shown promising results in the clinical trials.[54,55]

**Bioglass** Bioglass was developed to stimulate the formation of new bone.[56] It is used in orthopedics to cover the implants to promote union between implant and bone.[56,57] It has been used in dentistry to fill up the osseous defects during periodontal surgery.[58] It has been reported that a formulation of bioglass can promote infiltration and remineralization of dentinal tubules.[59] The basic component is silica, which acts as a nucleation site for precipitation of calcium and phosphate. SEM analysis has shown that bioglass application forms an apatite layer, which occludes the dentinal tubules.[59] The use of bioglass in management of DH has been shown by some products such as NovaMin (NovaMin Technology Inc., FL, USA). **Portland cement** Some authors have shown that calcium silicate cement derived from Portland cement can help in the management of DH.[17] It helps to occlude the dentinal tubules by remineralization. **Laser** Laser is an acronym for light amplification by stimulated emission of radiations. It has been shown in various studies that lasers can be used in the effective management of DH.[60–63] The mechanism of action of lasers in treating DH is not very clear. Some authors have shown that Nd–YAG laser application occluded the dentinal tubules.[61,62] GaAlA laser is thought to act by affecting the neural transmission in the dentinal tubules.[63] It has also been

proposed that lasers coagulate the proteins inside the dentinal tubules and block the movement of fluid.[61] Casein phosphopeptide–amorphous calcium phosphate Recently, milk protein casein has been used to develop a remineralizing agent (GC Tooth Mousse). The casein phosphopeptide (CPP) contains phosphoserine sequences which get attached and stabilized with amorphous calcium phosphate (ACP).[64] The stabilized CPP–ACP prevents the dissolution of calcium and phosphate ions and maintains a supersaturated solution of bioavailable calcium and phosphates.[64] Various studies have shown that CPP–ACP can effectively remineralize the enamel subsurface lesions.[65,66] By virtue of its remineralizing capacity, it has also been proposed by the manufacturers that it can also help in prevention and treatment of DH. MANAGEMENT STRATEGY • Take a detailed clinical and dietary history. • Differentiate and diagnose the condition from other dental pain conditions. • Identify and manage etiological and predisposing factors. • In case of mild-to-moderate sensitivity, advise at-home desensitizing therapy. • If there is no relief or in case of severe sensitivity, initiate in-office treatment. • In extreme cases, if patient does not respond to the therapy and there are individual teeth exhibiting the symptoms, then endodontic therapy can be initiated. • A regular review should be made with an emphasis on prevention of the condition.

#### Milk as Desensitizing Agent for Treatment of Dentine Hypersensitivity Following Periodontal Treatment Procedures

**ABSTRACT Background:** Dentinal hypersensitivity is a commonly observed problem after periodontal treatment procedures in periodontal patients. This further complicates preventive oral hygiene procedures by patients which jeopardize periodontal treatment, or even may aid in periodontal treatment failure. **Aims and Objectives:** The aims and objectives of present study were to assess the problem of dentine hypersensitivity after nonsurgical periodontal treatment and selection of cases for evaluation of commercially available milk at room temperature as mouth rinse for the treatment of dentinal hypersensitivity caused by periodontal treatment. **Materials and Methods:** Patients were selected randomly for nonsurgical periodontal treatment and then were assessed for dentine hypersensitivity. Those having dentine hypersensitivity were assigned in two groups. Group one patients were advised to rinse with commercially available milk at room temperature, group two patients were advised to rinse with luke warm water as control. A four point Verbal Rating Score (VRS) was designed to record the numerical value of dentine hypersensitivity. **results:** The results show incidence of 42.5% and prevalence of 77.5% for dentine hypersensitivity after periodontal treatment procedures. After rinsing with milk following periodontal treatment procedures, there was found a significant reduction of dentine hypersensitivity with probability by unpaired t-test as 0.0007 and 0.0001 at tenth and fifteenth day post periodontal treatment procedures respectively. **conclusion:** This study demonstrated that the milk rinse is a suitable, cheaper, fast acting, home-use and easily available solution to the problem of dentine hypersensitivity after nonsurgical periodontal treatment. Milk can be used as desensitizing agent and rinsing with milk for few days is effective in quick reduction of dentine hypersensitivity due to periodontal treatment procedures.

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can be due to toothbrush abrasion, pocket reduction surgery, tooth preparation for crown, excessive flossing or secondary to periodontal diseases [9]. For DH to occur, the lesion localization has to be initiated. Lesion initiation occurs after the protective covering of smear layer is removed causing exposure and opening of dentinal tubules, which culminates in lesion localization and DH [4]. Recent in the development of desensitizing agents, milk protein casein has been used to develop a remineralizing agent named GC Tooth Mousse (Recaldent, GC Corp, Japan). This milk protein casein phosphor-peptide (CPP) contains phosphoserine sequences which by attaching with amorphous calcium phosphate (ACP) of teeth forms stabilized CPP-ACP. This stabilized CPP-ACP prevents the dissolution of calcium and phosphate ions and maintains a supersaturated solution of bioavailable calcium and phosphates [4]. It has also been shown that this stabilized CPP-ACP can effectively remineralize the subsurface enamel lesions [4]. This remineralizing capacity of CPP can also help in prevention and treatment of DH [4]. Cai F et al., proposed in his study that incorporation of casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) into lozenges significantly increases enamel subsurface lesion remineralization [10]. S Lata et al., concluded that amorphous calcium phosphate- casein phosphopeptide (CPP-ACP) GC Tooth Mousse, Recaldent, GC Corp, Japan, in cream form is less effective than fluoride varnish for enamel subsurface remineralization [11]. Ruchi Vashisht et al., in ex-vivo study on artificial early enamel lesions concluded that casein phosphopeptide amorphous calcium phosphate has the significant potential to remineralize the early enamel lesions [12].

AIMS And OBjectIveS The Periodontal disease is very common in the general population and most of the patients seek treatment either in the form of

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StAtIStIcS ANAlYsIS The results were tabulated and analyzed using two tail paired Student's t-test for GA and GB patients and two tail independent t-tests (unpaired student's t-test) for G1 and G2 subjects using following internet website.

<http://www.physics.csbsju.edu/stats/ttest.html>

reSultS The result of paired Student's t-test and statistics for GA and GB patients with DH at baseline pretreatment procedure and at second day post-periodontal treatment procedure (visit I post procedure) are shown in [Table/Fig-1].

Scores of Dh

baseline Dh Pre-Periodontal Procedure (\*Ga)

Dh at second Day Post- treatment Procedure (Visit i) (\*Gb)

Number of Patients

% of Patients

Number of Patients

% of Patients Score 1 25 62.5 9 22.5 Score 2 15 37.5 4 10 Score 3 0 0 26 65 Score 4 0 0 1 2.5

Total 40 100 40 100 Incidence 42.5 Prevalence 77.5 Mean 1.38 2.48 95% confidence

interval for Mean 1.218 thru 1.532 2.195 thru 2.755 Standard Deviation 0.490 0.877

Median 1.00 3.00 Average Absolute Deviation from Median 0.375 0.575 t-value -9.35

Degree of Freedom 39 Probability <0.05 [table/Fig-1]: DH Analysis for GA & GB. \* GA=

Group A (Pre-procedure Patients group), GB= Group B (Post-procedure Patients group)

The results of the unpaired Student's t-test for G1 and G2 at 95% confidence interval are summarized in the [Table/Fig-2] as follows:

Parameters

Visit i (2nd Day)

Visit ii (4th Day)

Visit iii (10th Day)

Visit iv (15th Day) \*MeanG1 2.94 2.53 1.68 1.12 \*\*MeanG2 2.86 2.64 2.5 2.36 t-value 0.581 -0.620 -3.77 -8.29 Sdev 0.401 0.507 0.626 0.414 Probability 0.57 0.54 0.0007 0.0001

[table/Fig-2]: Follow up Visits for G1 and G2 Patients. Critical value at 95% level of significance for two tail t-test is 2.05 ( $p < 0.05$ ). \*MeanG1 – Mean of scores of group one (G1) at different visits. \*\*MeanG2 – Mean of scores of group two (G2) at different visits.

The original data analysis of the results for G1 and G2 patients groups is shown in [Table/Fig-3,4] as follows:

No. of Visits Score 1 Score 2 Score 3 Score 4 Visit I (2nd Day) 0 2 (11.77%) 14 (82.35%) 1 (5.88%) Visit II (4th Day) 0 8 (47.06%) 9 (52.94%) 0 Visit III (10th Day) 8 (47.06%) 7 (41.18%) 2 (11.77%) 0 Visit IV (15th Day) 15 (88.24%) 2 (11.77%) 0 0 [table/Fig-3]:

Group One (G1) Cases.

nonsurgical or surgical periodontal therapy. It was observed that after periodontal treatment procedures, patients usually start complaining of hypersensitive teeth which may make them reluctant for oral hygiene preventive procedures and further periodontal treatment as well. The consequence of it may result in poor periodontal health. The aims and objectives of this study were to assess the dentine hypersensitivity after non- surgical periodontal treatment procedure and selection of cases for evaluation of commercially available milk at room temperature as mouth rinse for treatment of dentine hypersensitivity following periodontal treatment procedures and finding a suitable, cheaper, fast acting, home-use and easily available solution to the problem.

**MATERIALS AND METHODS** The present study was conducted in specialty clinic of periodontics in College of Dentistry, Jazan University, Kingdom of Saudi Arabia. Periodontal specialist randomly selected the samples for thorough scaling and root planing for the study. Every selected case was having the diagnosis of moderate to severe chronic periodontitis. Systemically healthy patients were included in the study. Those with unrestored carious lesions and periodontal treatment in the last four month period were excluded from the study. Patients with current desensitizing therapy or had received professionally applied desensitizing treatment during four months prior to the study were also excluded. After obtaining informed consent, 40 patients were randomly selected and checked for baseline dentine hypersensitivity (DH) scores. These patients were assigned group A (GA). After performing non-surgical periodontal procedure (scaling and root planing), these same patients were assigned group B (GB) and again checked for DH on second day (Visit I) of post procedure. On the first visit (second day) after scaling and root planing, only 31 patients having dentine hypersensitivity were selected for the study and divided randomly into two groups namely group one (G1) and group two (G2). Seventeen patients were kept in G1 and fourteen in G2. Both group patients were instructed to return for follow up on 4th, 10th and 15th day post-treatment. Scores of dentine hypersensitivity at second day (visit-I) post treatment were considered as baseline for the study. At the first visit (second day) after treatment procedure, G1 patients were advised to rinse oral cavity with 30 ml commercially available milk at room temperature five times daily for five

minutes for fifteen days and, G2 patients were advised to rinse with 30 ml luke warm water for five minutes, five times a day for fifteen days. The patients were instructed not to eat/drink for 30 minutes after rinse and not to use any other desensitizing agents and/or pain killers, during the study period. A 4 point Verbal Rating Scale (VRS) was designed for this study. It is a clinical scale to find out the numerical values of the clinical problem of dentine hypersensitivity. It was described to find the values of dentine hypersensitivity as follows: Score 1 – No hypersensitivity – No discomfort to thermal changes after drinking water at room temperature or cold water. Score 2 – Mild hypersensitivity – Mild discomfort after drinking water at room temperature and cold water. Score 3 – Moderate hypersensitivity – Moderate discomfort after drinking water at room temperature but cannot drink cold water. Score 4 – Severe hypersensitivity – Pain after drinking water at room temperature. Pain on breathing. Cannot tolerate cold water (severe pain). We have used the term dentine hypersensitivity (DH) for the problem described, throughout in our manuscript.

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 No. of Visits Score 1 Score 2 Score 3 Score 4 Visit I (2nd Day) 0 2 (14.29%) 12 (85.72%) 0  
 Visit II (4th Day) 0 5 (35.72%) 9 (64.29%) 0 Visit III (10th Day) 0 7 (50%) 7 (50%) 0  
 Visit IV (15th Day) 0 9 (64.29%) 5 (35.72%) 0 [table/Fig-4]: Group Two (G2) Cases

Bar diagram representation tables: The results are depicted as bar diagram as follows [Table/Fig-5,6].

[table/Fig-5]: Cases with milk (G1)

[table/Fig-6]: Cases with Luke warm water (G2)

DISCUSSION Periodontal therapy in the form of nonsurgical and surgical procedure is common and patients usually report discomfort/pain immediately following these procedures. Post-periodontal treatment procedure, dentine hypersensitivity is a common clinical condition and complaint. Discomfort and sometimes pain caused by DH may refrain a person from establishing and maintaining adequate oral hygiene, which further may complicate oral health. Dentine hypersensitivity is characterized by a sharp pain or discomfort arising as an overt response to thermal, chemical or osmotic stimuli which cannot be explained as arising from any other disease or dental problems [1,13]. Dentine hypersensitivity is caused due to exposure of dentine after the enamel or cementum at the root surface has been lost by the treatment, underlying dental and gingival diseases or physiologic wear and tear of the teeth. Presence of gingival recession and loss of enamel exposes underlying cementum and dentine respectively which get abraded, worn or erode more quickly than enamel, as dentine and cementum have lower inorganic mineral contents than enamel so dentine abrades 25 times faster than enamel and cementum abrades 35 times faster [14]. B Von Triol et al., in their review described dentine hypersensitivity as a short and sharp, painful response to an external stimulus applied to exposed dentine (Chabanski & Gillam, Holland et al.,) [6,15,16]. B Von Troil found a prevalence of root sensitivity of 9-23% and 54-55% before and after periodontal therapy respectively and proposed that it occurs in approximately half of the patients following subgingival scaling and root planing and its intensity increases for a few weeks after therapy and decreases afterwards [6].

Dentine is a living tissue consisting of organic and inorganic components. Within the dentine, dentinal tubules run from the pulp to the outer dentinal surfaces. Brannstrom's

Hydrodynamic theory of fluid displacement within the dentinal tubules is the most accepted theory of dentinal hypersensitivity. The fluid movement within dentinal tubules stimulates the A-delta fibers resulting in the well localized sharp pain perceived as dentine hypersensitivity [6,17]. Over the years many treatment modalities of dentine hypersensitivity with varying outcomes have been reported but none provide definite conclusion as to which treatment is superior. They are either in-office procedures or self-administered for at home-use. These methods function through blocking the pain response either by occluding the dentinal tubules or by preventing the neural transmission. Even a large number of published studies are not able to provide a gold standard of treatment or product for treatment of dentinal hypersensitivity. DG Gillam and R Orchardson stated that the individuals with periodontal disease have higher prevalence of dentinal hypersensitivity after periodontal therapy such as scaling and root planing procedures as compared to those presenting with healthy mouths and gingival recession [18]. Tammaro et al., concluded that successful periodontal treatment can be accomplished through good oral hygiene self-care measures, and by nonsurgical and surgical periodontal therapies which have unwanted side effects including gingival recession, exposure of underlying dentine following root cementum denudation with the risk of experiencing tooth sensitivity [19]. Acidic foods and drinks can change the oral environment which can dissolve the newly created smear layer [20]. The purpose of this study was to assess the dentine hypersensitivity after non-surgical periodontal treatment (scaling and root planing) and clinically evaluate the effectiveness of milk rinse as a desensitizing agent for post scaling and root planing dentine hypersensitivity. The values of DH at first visit are shown in [Table/ Fig-1], which gives an estimate of 77.5% of DH after non-surgical periodontal therapy [Table/Fig-1]. This predicts about 42.5% incidence and 77.5% prevalence of DH after scaling and root planing procedure. Probability for patients of G1 is 0.0001 ( $p < 0.05$ ) at 15th day and a critical value of 2.05 at 95% confidence interval which strongly rejected null hypothesis and suggested strongly positive results for the treatment of DH with milk rinse [Table/Fig-2]. Therefore, it is evident from this study that at fifteenth day, the DH has reduced considerably (88.24% G1 cases has given score 1) and only 11.77% cases were having mild hypersensitivity (score 2) [Table/Fig-3] while at fifteenth day mild and moderate hypersensitivity cases from G2 patients were considerably high (64.29% and 35.72% respectively) [Table/Fig-4]. Milk is a mixture of emulsions, colloids, molecular and ionic solutions. Fresh milk has a pH of 6.7 and is slightly acidic. Milk is also an excellent buffering solution which can resist a change in pH on addition of acid or alkali. When the pH of milk is changed, the acidic or the basic groups of the milk proteins will be neutralized. With the fall of the pH of milk, the charge on the casein also falls and it precipitates. Milk comprises less than 1% salts mainly in the form of chlorides, phosphates, citrates of calcium, sodium and magnesium. Calcium, magnesium, phosphorus and citrates are distributed between the soluble and colloidal phases. Equilibria of salts in milk are altered by heating, cooling and change in pH. Milk also contains various enzymes like phosphatases, lipases, peroxidases and catalases etc [21]. It is proposed from this study that scaling and root planning may itself create a smear layer that could be supplemented by natural dentinal tubules mineralization process through frequent milk rinsing. Rinsing with milk may provide the rich bioavailability of calcium and phosphate which can aid in remineralization, acid buffer and reduce the acidic effect of plaque on tooth structure. Milk also contains

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immunoglobulins [22], which may provide defenses against plaque microorganisms. Milk rinse could be an effective, suitable, cheaper, fast acting, easily available and home use substitute for dentine hypersensitivity. Thus, the results of this study suggested and indicated that dentine hypersensitivity in most of the cases occurs invariably after nonsurgical periodontal treatment and rinsing with milk is very effective in reducing the dentine hypersensitivity after periodontal treatment procedures. This study clearly establishes the desensitizing therapeutic benefit of milk rinsing following non-surgical periodontal treatment. Further studies are required to know the mechanism of milk rinse in prevention and treatment of dentine hypersensitivity. There are no other comparable studies available for milk rinse in the treatment of dentine hypersensitivity after periodontal treatment.

coNcluSIOn The present study demonstrated clearly that the rinsing with 30 ml milk at room temperature five times daily for few days is effective in reducing dentine hypersensitivity after periodontal treatment procedures. Milk is a suitable, cheaper, fast acting, home-use and easily available solution to the problem of dentine hypersensitivity and can be used as desensitizing agent for dentine hypersensitivity due to periodontal treatment procedures.

A comparative evaluation of propolis and 5.0% potassium nitrate as a dentine desensitizer: A clinical study Aamir Rashid Purra, Mubashir Mushtaq, Shashi Rashmi Acharya,<sup>1</sup> and Vidya Saraswati<sup>1</sup> Department of Conservative Dentistry and Endodontics, Government Dental College and Hospital Srinagar, Kashmir, India <sup>1</sup>Department of Conservative Dentistry and Endodontics, Manipal College of Dental Sciences, Manipal, India Address for correspondence: Dr. Aamir Rashid Purra, Department of Conservative Dentistry and Endodontics, Government Dental College and Hospital Srinagar, Kashmir, India. E-mail: moc.liamtekcor@odnerihsum Received 2013 Sep 15; Accepted 2013 Dec 25. Copyright : © Journal of Indian Society of Periodontology This is an open-access article distributed under the terms of the Creative Commons Attribution Noncommercial-Share Alike 3.0 Unported, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Abstract Aim: The purpose of this clinical study was to evaluate the efficacy of saturated ethanolic solution of Propolis for the treatment of dentin hypersensitivity. Materials and Methods: Ten patients aged 20-40 years with 156 hypersensitive teeth were selected for a 3-month study. Each patient was

subjected to treatment with saturated ethanolic solution of Propolis, 5% potassium nitrate and distilled water. The patients were recalled at seventh day, 2 weeks and 4 weeks for the application of the agent and re-evaluation. The final re-evaluation of the patients was done after 3 months from the first application. The responses of the patients to the test temperatures were converted to a ranking and data was statistically analyzed. A statistical analysis was done using ANOVA and Bonferroni test and Tukey HSD test for multicomparison. Results: The results between the Propolis group and the potassium nitrate group showed no significant difference in the immediate post-treatment period; however, the results were significant at the end of first week and second week. At 4 weeks and 3 months period, a comparison between the groups again showed no significant difference. Conclusion: It was concluded that Propolis was more effective than 5% potassium nitrate in relieving dentinal hypersensitivity and had an immediate and sustained effect. Keywords: Cold graded water test, distilled water, potassium nitrate, propolis

**INTRODUCTION** Dentin hypersensitivity is one of the most painful and least successfully treated chronic problems of the teeth. It is one of the common complaints and has been reported that as many as one in every seven patients undergoing dental treatment experiences this painful condition.[1] A wide array of treatment modalities is available for the management of dentin hypersensitivity. These modalities involve the application of various chemicals (desensitizing agents) such as potassium or ferric oxalates, potassium nitrate, stannous fluoride, sodium fluoride, sodium monofluorophosphate (MFP), strontium chloride, copal varnishes, calcium hydroxide, fluoride treatments, dentin bonding agents, or iontophoresis. The desensitizing agents are applied either by the dentist (in office treatment) or used by the patient as home application. Home applications are mainly in the form of dentifrices and also as mouthwashes. The effects of home-applied agents are manifested after a period of time and would require a considerable degree of patient compliance. In office treatment, modalities provide instantaneous relief to the patient, but the effects are often temporary. Thus, none of the treatment modalities has been able to provide a permanent relief from dentin hypersensitivity. Potassium nitrate was introduced as a desensitizing agent by Hodosh.[2] It has been one of the widely prescribed desensitizing agents. It has been used in concentrations of 1%, 2%, 5%, 10%, and 15% or as a saturated solution. 5% is said to be most effective and is also incorporated into dentifrices.[3] Potassium nitrate is supposed to reduce hypersensitivity by inducing a sustained depolarization of nerve membrane as put forward by Pashlay.[4] It has also been postulated that blockage of dentinal tubules by potassium nitrate reduces sensitivity.[5]

Propolis is a resinous yellow brown to dark brown substance collected by honey bees from sprouts, exudates of trees and other parts of plants and modified in the beehives by addition of salivated secretions and wax. It is used by bees for protection, to repair openings and damages in hives, to construct aseptic places for queen egg and to embalm killed invaders. Chemically, Propolis of different parts of the world is constituted by 50-60% of resins, 30-40% of waxes, 5-10% of essential oils, 5% pollen, besides microelements like aluminum and calcium.[6] So far, more than 300 organic compounds of different groups mainly phenolic, such as: Flavonoids, stilbenes, phenolic acids and its esters have been identified from Propolis. Many biological activities have been reported for Propolis, such as antimicrobial, antiparasitic, antiviral, antiinflammatory, antitumor, antioxidant, anesthetic and free

radical scavenging action.[7] A pioneering pilot study was under taken on the effect of Propolis on dentinal hypersensitivity in vivo for a study period of 4 weeks.[8] It was concluded that Propolis had a positive effect in control of dentin hypersensitivity. Thus, the primary objective of this 3-month study was to evaluate the clinical efficacy of topical Propolis preparation and to compare it with a topical 5% potassium nitrate preparation in the treatment of dentin hypersensitivity using distilled water as a control.

**MATERIALS AND METHODS** The subjects included in this study were selected from outpatients attending the Department of Conservative Dentistry and Endodontics. The study was designed following consideration of the 1997 guidelines on clinical trials of dentine sensitivity.[9,10] The study was a 3-month, randomized, double blind study. The subjects fulfilling certain qualifying criteria were taken up for the study. (Subjects included in the study were dentally mature individuals in the age group of 20-40 years, having sensitive teeth with a clinical diagnosis satisfying the definition of dentine hypersensitivity; subjects willing to participate in the study for 3 months; subjects with more than three hypersensitive teeth and the subjects capable of giving a written informed consent. Subjects excluded from the study included the patients who were on current desensitizing therapy, patients with medical (including psychiatric) and pharmacotherapeutic histories that may compromise the protocol-including the use of anti-inflammatory, analgesic and mindaltering drugs; patient who were pregnant or breast feeding; patients with allergies, idiosyncratic reactions or with eating disorders; patients with systemic conditions that are etiologic or predisposing to dentine hypersensitivity (e.g., chronic acid regurgitation); patients with excessive dietary or environmental exposure to acids; patients with the history of Periodontal surgery in the preceding 3 months (unless it is the effect of the agent on post-surgical sensitivity that is under study); patients with orthodontic appliance treatment with in previous 3 months; patients with painful pathology or defects in teeth or their supporting structures; patients who had their teeth restored in the preceding 3 months; patients with fixed or removable prostheses or extensively restored teeth with restorations extending into the test area.

Ten patients in the age group of 20-40 years with 156 hypersensitive teeth fulfilling the abovementioned criteria were included in the study. The teeth selected for the study included canine, premolars and molars. Relevant information related to history, precipitating causes, and past treatment received for the condition was collected from each patient.

Thorough oral prophylaxis was done for all the patients. Following this, the patients were allocated to treatment. The agents were assigned as 5% potassium nitrate solution, alcoholic solution of Propolis (Hi-Tech Natural Products, India Ltd) and distilled water. Propolis extract was made by dissolving the Propolis into 70% ethanol and straining out the precipitate. The study was carried out in a double-blinded design, i.e., the patient, and the people analyzing the data were unaware of the agent. One experienced operator was involved in the application and other in the evaluation of the agent.

Freshly prepared potassium nitrate was used at each appointment. Based on the treatment received, the hypersensitive sites were divided into three groups:

- Group 1 (Propolis)
- Group 2 (5% potassium nitrate)
- Group 3 (distilled water).

Fifty-two hypersensitive teeth were allocated in each group. Each patient received all the three treatments. The area to be treated was isolated with rubber dam and was dried using air

spray. Propolis was applied with a brush and left undisturbed at the site for 5 minutes. Potassium nitrate and water was applied with saturated cotton pellets for 5 minutes. Patients were instructed not to rinse or to take anything for half an hour so that the desensitizing agent takes sufficient time to act without getting washed away. The patients were advised to use a soft toothbrush and a nonfluoridated tooth paste while brushing. Dietary counseling was also done in order to avoid intake of excessive dietary acids during the study period.

Pre- and post-treatment assessment was done at the baseline, 7th day, 14th day, 30th day, 3 months and measurements were recorded by the same investigator. Evaluation of hypersensitivity The subject's sensitivity to stimuli was assessed by using a cold graded thermal test. The clinical evaluation of the sensitivity was done before and after the application of the agents at 1st, 7th, 14th, 30th day and at 3 months recall. A thermal testing technique developed by Brough et al. was used to quantify the patient's baseline response to a cold stimulus.[11] Each tooth included in the study was isolated with a rubber dam without a clamp, using finger adaptation only. The water temperatures used in this study were 20°C, 10°C and 0°C with a variance of +1°C for each temperature interval. Water temperature was maintained throughout the procedure by the use of thermal-insulated containers. The required water temperature was adjusted in each container by the addition of ice or hot water until the desired temperature was obtained. The water temperature was monitored by the use of a thermometer. The application of water was done using a disposable syringe. The syringe was changed after every patient. The syringes were kept in the water containers and remained there until used to transport and apply water to the exposure site. Each syringe was immediately returned to the container following use. Using the syringe and starting at 20°C the investigator flowed the water over the isolated exposed tooth surface until a sensitive response occurred or for a maximum of 3 s, if no response occurred.

The investigator waited for 2 minutes and then retested the tooth with water at 10°C. Delaying reapplication of water for 2 minutes between each application was attempted to allow the tooth to return to body temperature. The water temperature was finally decreased by 10°C intervals until a sensitive response was obtained or until the testing system's limit (0°C) was reached. As the thermal stimulus was applied, the thermal stimulus either will or will not elicit a sensitive response (yes/no). Following baseline data collection, the appropriate solutions, according to the treatment group assignment of each tooth, were applied by the examiner. The facial surface was dried with gauze before the application of the treatment solutions. The investigator used a brush saturated with the treatment solution for Propolis and gauzes saturated with treatment solution for potassium nitrate and water and carefully placed them without burnishing on the exposed tooth surface for 2 minutes. The investigator again dried the tooth with gauze and immediately applied similarly the second coat of solution for an additional 3 minutes. After the tooth had been treated, the investigator determined the patient's immediate response to water temperatures using the identical procedure that was performed at baseline. Each tooth was treated and evaluated individually before proceeding to the next one. After all the teeth had been tested, the patient was instructed to use normal daily oral hygiene regimen and to avoid using any desensitizing dentifrices or any agents that contained fluoride. The teeth were again treated with agents at 7th day, 14th day and 28th day. Additional post-treatment

measurements at 7th day, 14th day, 28th day and 3 months after initial treatment were made using the identical thermal testing technique used to establishing baseline and immediate response data. RESULTS Ten subjects with 156 hypersensitive teeth completed the 3-month clinical study.

For purpose of data analysis the responses of the patients to the test temperatures were converted to a ranking as suggested by Brough et al.[11] The ranking were assigned as follows:

• 3 = 20°C • 2 = 10°C • 1 = 0°C • 0 = No response.

The effectiveness of Propolis in reducing dentine hypersensitivity was compared with 5% potassium nitrate and placebo treatment at baseline, 7th day, 2 weeks, 4 weeks and 3 month.

In Group 1, out of 52 teeth, 20 teeth reacted at 20°C, 32 teeth at 10°C before treatment as shown in Table 1. In Group 1, application of Propolis resulted in significant reduction in the temperature to which the patient responded immediately after treatment, at 1 week, 2 weeks, 4 weeks and 3 month after treatment. At the end of 3 months only 7 teeth reacted at 20°C, 9 teeth reacted at 10°C, 9 teeth react at 0°C and 27 teeth showed no response i.e., 51.9% teeth were completely free of sensitivity.

In Group 2 out of 52 teeth, 23 reacted at 20°C and 29 reacted at 10° before treatment as shown in Table 2. In Group 2, application of 5% potassium nitrate resulted in a significant reduction in the temperature to which the patient responded at 1 week, 2 weeks, 4 weeks and 3 month after treatment. At the end of 3 months only 12 teeth reacted at 20°C, 12 teeth reacted at 10°C, 13 teeth reacted at 0°C and 15 teeth showed no response i.e., 28.8% teeth were completely free of sensitivity.

In Group 3 out of 52 teeth, 24 reacted at 20°C and 28 reacted at 10°C before treatment as shown in Table 3. In Group 3, who were treated with placebo treatment, slight reduction in the temperature to which the patient responded after treatment was seen but it was not statistically significant at 1 week, 2 weeks and 4 weeks after the treatment. At the end of 3 months a significant response was seen. 17 teeth were reacting at 20°C, 20 teeth were reacting at 10°C, 7 teeth were reacting at 0°C, and 8 teeth showed no response.

To obtain intra-group and inter-group comparisons, mean values with standard deviations for different time periods in Groups 1, 2 and 3 were statistically analyzed. P value was set at 0.05 as shown in Table 4 and following results were obtained.

A statistical analysis was done using ANOVA and multicomparison was done using the Bonferroni test and Tukey HSD test. Intragroup comparison When the results were compared in intra group at baseline, immediately after treatment, 1 week, 2 weeks, 4 weeks and 3 months using the Bonferroni test, there was a significant difference. In Group 1, on comparison between baseline and immediately after treatment, 1 week, 2 weeks, 4 weeks and 3 months, the results were very highly significant ( $P = 0.001$ ). On comparison between immediately after treatment and 1 week, the result was significant. A comparison between results immediately after treatment and 2 weeks was highly significant, with 4 weeks was not significant but was significant with 3 months. Rest of the comparisons were not significant. In Group 2, a comparison between baseline and immediately after treatment, 1 week, 2 weeks and 3 months were very highly significant as shown. On comparison between immediately after treatment and 1 week results were not significant, with 2 weeks results were significant, with 4 weeks results were highly significant but not significant with 3 months. Rest of the comparisons were not significant. In Group 3,

comparisons of results between different time periods were not significant except between baseline and 3 months. Intergroup comparison When baseline score of all the groups were compared with each other using the Tukey HSD test, there was no significant difference found ( $P > 0.05$ ) as shown in Table 5.

Immediately after the treatment, the results between the groups showed that there was no significant difference between Group 1 and Group 2 and Group 2 and Group 3 but between Group 1 and Group 3 results were highly significant. A comparison at first week between groups showed that there was a significant difference between Group 1 and Group 2 and very highly significant difference between Group 1 and Group 3. Group 2 and Group 3 showed highly significant difference. At 2 weeks a comparison between groups showed same results as at first week. At 4 weeks a comparison between groups showed that there was no significant difference between Group 1 and Group 2 but there was very highly significant difference between Group 1 and Group 3 and also between Group 2 and Group 3. At 3 months a comparison between Group 1 and Group 3 was very highly significant but a comparison between Group 1 and Group 2 as well as Group 2 and Group 3 was insignificant.

**DISCUSSION** It has been reported that 15% of global population suffers from dentine hypersensitivity.[12] Till now no completely reliable treatment has been found; an agent can be of benefit in one case but fail in another. Search for an ideal agent still continues. Natural products have been used for thousands of years in folk medicine for several purposes. Among them, Propolis a bee product has attracted increased interest due to its harmless nature and innumerable biological activities. Certain clinical studies have shown Propolis to be a promising dentine desensitizer in the treatment of dentinal hypersensitivity.[8] The authors explained the effect due to blockade of dentinal tubules in light of their SEM observation.[9] Literature review indicates that there has been no study comparing Propolis with a known desensitizing agent and a negative control.

The aim of this study was to evaluate the clinical efficacy of Propolis in a controlled study and compare it with a positive and a negative control. Potassium nitrate was used as a positive control as it is currently the most frequently used agents for control of hypersensitivity.[13] A purified saturated ethanolic extract of Propolis was used in the study.

One of the inherent problems of desensitizing studies is in the development of a technique and stimulus that will quantify reliably a patient's response to pain Only in few studies were fairly sophisticated thermoelectric measuring devices used.[14] These devices give a precise reading but are extremely expensive. In the present study, the Brough technique of using cold graded water enabled the investigator to confine the stimulus to the tooth being tested, was reproducible, inexpensive and could stimulate the irritation of a cold stimulus within a realistic temperature range.[11] As the thermal stimulus was applied, the temperature at which sensitivity was elicited was noted using the pain scoring system of Brough et al.[11] According to the system the thermal stimulus either will or will not elicit a sensitive response (Yes/No). They found that by asking the participant simply to report that the tooth was or was not painful eliminated the requirement that the patient classify the pain response e.g., mild, moderate, severe or very severe. This enabled the examiner to collect quantitative data and allowed for statistical analysis. This technique determines the degree of hypersensitivity through the use of a range of water temperatures applied directly to the exposed root surface.

Application of Propolis in Group 1 resulted in a significant reduction in the temperature to which the patient responded immediately after treatment in 19% of teeth and 25% teeth responded only at 0°C 5 minutes after treatment. This was followed by an increase in the efficacy of agent over a period of time with maximum relief from hypersensitivity by end of the study period i.e., 3 months. At 3 months after the application of the agent, 51% teeth very completely free of sensitivity and those that reacted only at 0°C decreased to 17.3%. Propolis thus had an immediate as well as increased sustained effect. The immediate relief could be due to its tubular sealing effect which prevents the flow of the dentinal fluid in the tubules, thereby preventing any alteration in the arrangement of the odontoblastic process and nerve endings. The long lasting of effect of Propolis probably could be due to stable nature of the deposits so formed. The retention and stability of the Propolis deposits in hostile oral environment needs further investigation and is not within the scope of this study. Over 3 months time response gradually reduced to only 28.8% teeth free from hypersensitivity and 25% teeth reacting at 0°C. Propolis is a powerhouse of chemicals especially bioactive flavonoids. Flavonoids have been shown to have tissue regenerative activity. Propolis has been shown to stimulate various enzymes, cell metabolism, circulation and collagen formation, as well as improve healing.[15] Sabir et al. found that direct pulp capping with Propolis flavonoids in rats may delay dental pulp inflammation and stimulate reparative dentin formation.[16] In our study no histologic sections were taken to determine if Propolis formed reparative dentine, but this is a possible explanation for the decrease in sensitivity at the 3-month interval. Potassium nitrate acts by reducing the excitability of the interdental nerves in the pulp by depolarizing them.[17,18] Potassium ions have to traverse the length of the dentinal tubule in sufficient quantity to cause depolarization. The results show that at the end of 5 minutes after treatment, the concentration of potassium ions in the dentinal tubules was not sufficient to make the nerves excitable and it takes longer time for the potassium ions to reach the nerves. The effect of desensitization achieved by K ions decreased with time and this could be attributed to a decrease in concentration of the ions in dentinal tubules. The placebo treatment which was used in Group 3 was effective only to a certain extent. With 9.6% teeth free from sensitivity and 7.7% reacting at 0°C only, 5 minutes after treatment and increasing to 15.3% teeth free from sensitivity and 13.5% reacting at 0°C at the end of the study. Placebo effect consists of a complex mixture of physiologic and psychological interactions, depending considerably on the doctor patient relationship with both parties believing that the treatment is of value and the desire to obtain relief from symptoms. Despite randomization of subjects in the study, the enrolled subjects often try to please clinicians. Furthermore, positive emotional and motivational behavioral responses can activate the body's central pain inhibiting system, which can modulate painful stimuli from the periphery through the release of endorphins centrally. Yet another possible phenomenon which could occur is the 'Hawthorne effect'.[19] This may influence the interpretation of the results. The 'Hawthorne effect' is a response to nonintervention procedure such as frequent examinations, improved oral hygiene or compliance with the treatment regimen. Patients frequently appear to improve merely from the effect of being placed on a trial. This reaction is thought to occur in many clinical trials. In this study, patients were reinforced in each visit about their oral hygiene and dietary acid consumption. Further, most patients like to have clean mouths if they attend the dental surgeon and will therefore be more vigilant about brushing during a period of frequent

examination. These factors unintentionally alter behavior. Better oral hygiene may allow greater saliva access to patent dentinal tubules. This in turn may enhance tubules obliteration through deposition of salivary calcium, phosphate and proteins. The influence of Hawthorne effect may explain the change in the results from 5 minutes after treatment to end of study at 3 months. Many investigators have described patients obtaining relief without treatment due to placebo effect.[19,20] This is thought to vary from 20% to 60% in dentine sensitivity clinical trials.[21] CONCLUSION Observations from the present study are:

- Propolis was the most effective desensitizer providing both immediate relief which gradually increased by the end of 3 months
- 5% potassium nitrate was effective in reducing dentin hypersensitivity but to a lesser degree when compared to Propolis
- The placebo group failed to show any statistically significant difference than the other experimental agents and was not effective in reducing dentin sensitivity.

48. Dental problems usually cause local issues with soft and hard tissues but rarely cause systemic complications.

- a. True
- b. False

## Systemic diseases and oral health.

The US population is at the beginning of a significant demographic shift; the American geriatric population is burgeoning, and average longevity is projected to increase in the coming years. Elder adults are affected by numerous chronic conditions, such as diabetes, hypertension, osteoarthritis, osteoporosis, cardiovascular diseases, and cerebrovascular diseases. These older adults need special dental care and an improved understanding of the complex interactions of oral disease and systemic chronic diseases that can complicate their treatment. Oral diseases have strong associations with systemic diseases, and poor oral health can worsen the impact of systemic diseases.

Everything You Need to Know About Dental and Oral Health Overview Dental and oral health is an essential part of your overall health and well-being. Poor oral hygiene can lead to dental cavities and gum disease, and has also been linked to heart disease, cancer, and diabetes.

Maintaining healthy teeth and gums is a lifelong commitment. The earlier you learn proper oral hygiene habits — such as brushing, flossing, and limiting your sugar intake — the easier it'll be to avoid costly dental procedures and long-term health issues. Facts about dental and oral health

Dental cavities and gum disease are very common. According to the World Health Organization Trusted Source: • between 60 and 90 percent of school children have at least one dental cavity • nearly 100 percent of adults have at least one dental cavity • between 15 and 20 percent of adults ages 35 to 44 have severe gum disease • about 30 percent of people around the world ages 65 to 74 don't have any natural teeth left • in most countries, out of every 100,000 people, there are between 1 and 10 cases of oral cancer • the burden of oral disease is much higher in poor or disadvantaged population groups

There are many steps you can take to keep your teeth healthy. For example, dental and oral disease can be greatly reduced by:

- brushing your teeth with fluoride toothpaste at least twice a day
- flossing your teeth at least once a day
- decreasing your intake of sugar
- eating a diet high in fruits and vegetables
- avoiding tobacco products
- drinking fluoridated water
- seeking professional dental care

Symptoms of dental and oral problems You shouldn't wait until you have symptoms to visit your dentist. Going to the dentist twice a year will usually allow them to catch a problem before you even notice any symptoms.

If you experience any of the following warning signs of dental health issues, you should make an appointment to see your dentist as soon as possible:

- ulcers, sores, or tender areas in the mouth that won't heal after a week or two
- bleeding or swollen gums after brushing or flossing
- chronic bad breath
- sudden sensitivity to hot and cold temperatures or beverages
- pain or toothache
- loose teeth
- receding gums
- pain with chewing or biting
- swelling of the face and cheek
- clicking of the jaw
- cracked or broken teeth
- frequent dry mouth

If any of these symptoms are accompanied by a high fever and facial or neck swelling, you should seek emergency medical treatment. Learn more about the warning signs of oral health issues.

Causes of dental and oral diseases Your oral cavity collects all sorts of bacteria, viruses, and fungi. Some of them belong there, making up the normal flora of your mouth. They're generally harmless in small quantities. But a diet high in sugar creates conditions in which acid-producing bacteria can flourish. This acid dissolves tooth enamel and causes dental cavities.

Bacteria near your gumline thrive in a sticky matrix called plaque. Plaque accumulates, hardens, and migrates down the length of your tooth if it isn't removed regularly by brushing and flossing. This can inflame your gums and cause the condition known as gingivitis.

Increased inflammation causes your gums to begin to pull away from your teeth. This process creates pockets in which pus may eventually collect. This more advanced stage of gum disease is called periodontitis.

There are many factors that contribute to gingivitis and periodontitis, including:

- smoking
- poor brushing habits
- frequent snacking on sugary foods and drinks
- diabetes
- the use of medications that reduce the amount of saliva in the mouth
- family history, or genetics
- certain infections, such as HIV or AIDS
- hormonal changes in women
- acid reflux, or heartburn

- frequent vomiting, due to the acid

Diagnosing dental and oral diseases Most dental and oral problems can be diagnosed during a dental exam. During an exam, your dentist will closely inspect your:

- teeth • mouth • throat • tongue • cheeks • jaw • neck

Your dentist might tap or scrape at your teeth with various tools or instruments to assist with a diagnosis. A technician at the dentist's office will take dental X-rays of your mouth, making sure to get an image of each of your teeth. Be sure to tell your dentist if you're pregnant. Women who are pregnant shouldn't have X-rays.

A tool called a probe can be used to measure your gum pockets. This small ruler can tell your dentist whether or not you have gum disease or receding gums. In a healthy mouth, the depth of the pockets between the teeth are usually between 1 and 3 millimeters (mm). Any measurement higher than that may mean you have gum disease.

If your dentist finds any abnormal lumps, lesions, or growths in your mouth, they may perform a gum biopsy. During a biopsy, a small piece of tissue is removed from the growth or lesion. The sample is then sent to a laboratory for examination under a microscope to check for cancerous cells. If oral cancer is suspected, your dentist may also order imaging tests to see if the cancer has spread.

Tests may include:

- X-ray • MRI scan • CT scan • endoscopy

Types of dental and oral diseases We use our teeth and mouths for a lot, so it's not surprising how many things can go wrong over time, especially if you don't take proper care of your teeth. Most dental and oral problems can be prevented with proper oral hygiene. You'll likely experience at least one dental problem during your lifetime.

**Cavities** Cavities are also called caries or tooth decay. These are areas of the tooth that have been permanently damaged and may even have holes in them. Cavities are fairly common. They occur when bacteria, food, and acid coat your teeth and form a plaque. The acid on your teeth starts to eat away at the enamel and then the underlying dentin, or connective tissue. Over time, this can lead to permanent damage.

**Gum disease (gingivitis)** Gum disease, also called gingivitis, is inflammation of the gums. It's usually the result of plaque building up on your teeth due to poor brushing and flossing habits. Gingivitis can make your gums swell and bleed when you brush or floss. Untreated gingivitis can lead to periodontitis, a more serious infection.

**Periodontitis** As periodontitis progresses, the infection can spread to your jaw and bones. It can also cause an inflammatory response throughout

the body. Cracked or broken teeth A tooth can crack or break from an injury to the mouth, chewing hard foods, or grinding the teeth at night. A cracked tooth can be very painful. You should visit your dentist right away if you've cracked or broken a tooth. Sensitive teeth If your teeth are sensitive, you might feel pain or discomfort after having cold or hot foods or beverages.

Tooth sensitivity is also referred to as "dentin hypersensitivity." It sometimes occurs temporarily after having a root canal or a filling. It can also be the result of:

- gum disease • receding gums • a cracked tooth • worn-down fillings or crowns

Some people naturally have sensitive teeth because they have thinner enamel.

Most of the time, naturally sensitive teeth can be treated with a change in your daily oral hygiene regimen. There are specific brands of toothpaste and mouthwash for people with sensitive teeth.

Shop for toothpaste and mouthwash made for people with sensitive teeth.

Oral cancer Oral cancers include cancer of the:

- gums • tongue • lips • cheek • floor of the mouth • hard and soft palate

A dentist is usually the first person to recognize oral cancer. Tobacco use, such as smoking and chewing tobacco, is the biggest risk factor for oral cancer. According to the Oral Cancer Foundation (OCF), nearly 50,000 Americans will be diagnosed with oral cancer this year. In general, the earlier that oral cancer is diagnosed, the better the outlook. The link between oral and general health Oral health has risen in importance in recent years, as researchers have discovered a connection between declining oral health and underlying systemic conditions. It turns out that a healthy mouth can help you maintain a healthy body. According to the Mayo Clinic, oral bacteria and inflammation may be associated with:

- heart disease • endocarditis, or inflammation of the lining of the heart • premature birth • low birth weight

Bacteria can spread from your oral cavity to your bloodstream, causing infective endocarditis.

Infective endocarditis is a life-threatening infection of your heart valves. Your dentist may suggest you take antibiotics as a preventive measure before they perform any dental procedure that could dislodge bacteria in your mouth. Treating dental and oral problems Even if you've been taking good

care of your teeth, you'll still need to have a professional cleaning twice a year during a routine visit with your dentist. Your dentist will recommend other treatments if you show signs of gum disease, infections, or other problems. Cleanings A professional cleaning can get rid of any plaque you may have missed while brushing and flossing. It'll also remove tartar. These cleanings are usually performed by a dental hygienist.

After all the tartar is removed from your teeth, the hygienist will use a high-powered toothbrush to brush your teeth. This is followed by flossing and rinsing to wash out any debris.

A deep cleaning is also known as scaling and root planning. It removes tartar from above and below the gumline that can't be reached during a routine cleaning. Fluoride treatments Following a dental cleaning, your dentist may apply a fluoride treatment to help fight off cavities. Fluoride is a naturally occurring mineral. It can help strengthen the enamel of your tooth and make them more resilient to bacteria and acid. Antibiotics If you show signs of a gum infection or you have a tooth abscess that has spread to other teeth or your jaw, your dentist may prescribe antibiotics to help get rid of the infection. The antibiotic may be in the form of a mouth rinse, gel, oral tablet, or capsule. Topical antibiotic gel may also be applied to the teeth or gums during surgical procedures. Fillings, crowns, and sealants A filling is used to repair a cavity, crack, or hole in the tooth. The dentist will first use a drill to remove the damaged area of the tooth and then fill the hole with some material, such as amalgam or composite.

A crown is used if a large portion of your tooth needs to be removed or has broken off due to an injury. There are two types of crowns: an implant crown that fits over an implant, and a regular crown that fits over a natural tooth. Both types of crowns fill in the gap where your natural tooth appeared.

Dental sealants are thin, protective coatings that are placed on the back teeth, or molars, to help prevent cavities. Your dentist may recommend a sealant for your children as soon as they get their first molars, at around age six, and again when they get their second set of molars around age 12. Sealants are easy to apply and completely painless. Root canal You might need a root canal if tooth decay reaches all the way inside the tooth to the nerve. During a root canal, the nerve is removed and replaced with a filling made of a biocompatible material, usually a combination of a rubber-like material called gutta-percha and adhesive cement. Probiotics Probiotics are mostly known for their role in digestive health, but new research has shown that the healthy bacteria may be beneficial for your teeth and gums.

Probiotics have been shown to prevent plaque and treat bad breath. They also help to prevent oral cancers and decrease inflammation from gum disease.

While large clinical trials are still needed to prove their effectiveness, results to date have been promising. You can take a probiotic supplement or eat foods high in beneficial bacteria, such as yogurt, kefir, and kimchi. Other popular probiotic foods include sauerkraut, tempeh, and miso.

Changing daily habits Keeping your mouth healthy is a daily commitment. A dental hygienist can teach you how to properly take care of your teeth and gums on a daily basis. In addition to brushing and flossing, your daily routine can include mouthwash, oral rinses, and possibly other tools, such as a Waterpik water flosser.

Shop for a water flosser.

HEALTHLINE RESOURCES Find the diet that's right for you with our free diet quiz

Our free assessment ranks the best diets for you based on your answers to 3 quick questions.

Surgery for dental and oral problems Oral surgeries are usually performed to treat more serious cases of periodontal disease. Certain dental surgeries can also be done to replace or fix missing or broken teeth caused by an accident.

Flap surgery During a flap surgery, a surgeon makes a small cut in the gum to lift up a section of the tissue. They then remove tartar and bacteria from underneath the gums. The flap is then stitched back into place around your teeth.

Bone grafting Bone grafting is needed when gum disease causes damage to the bone surrounding the root of your tooth. The dentist replaces the damaged bone with a graft, which can be made from your own bone, a synthetic bone, or a donated bone.

Soft tissue grafts A soft tissue graft is used to treat receding gums. A dentist will remove a small piece of tissue from your mouth or use a donor tissue and attach it to the areas of your gums that are missing.

Tooth extraction

If your dentist can't save your tooth with a root canal or other surgery, the tooth will likely need to be extracted.

You may also need a tooth extraction if your wisdom teeth, or third molars, are impacted. Sometimes, a person's jaw isn't large enough to accommodate the third set of molars. One or more of the wisdom teeth will become trapped or impacted when it tries to emerge. A dentist will typically recommend that wisdom teeth be extracted if they cause pain, inflammation, or other problems.

Dental implants Dental implants are used to replace missing teeth that are lost due to a disease or

an accident. An implant is surgically placed into the jawbone. After the implant is placed, your bones will grow around it. This is called osseointegration.

Once this process is complete, your dentist will customize a new artificial tooth for you that matches your other teeth. This artificial tooth is known as a crown. The new crown is then attached to the implant. If you're replacing more than one tooth, your dentist may customize a bridge to fit into your mouth. A dental bridge is made of two abutment crowns on either side of the gap, which then hold the artificial teeth in between in place. What can go wrong? Periodontal disease can eventually break down the bone that supports your teeth. This can lead to many complications. You'll likely need dental treatment to save your teeth.

Risks and complications of untreated periodontal disease include:

- tooth abscesses
- other infections
- migration of your teeth
- pregnancy complications
- exposure of the roots of your teeth
- oral cancer
- tooth loss
- increased risk of diabetes, heart disease, cancer, and respiratory diseases

If left untreated, an infection from a tooth abscess can spread to other parts of your head or neck. It can even lead to sepsis, a life-threatening blood infection. Keeping your teeth and gums healthy Good oral health boils down to good general health and common sense. The best ways to prevent oral health problems are to:

- brush your teeth with fluoride toothpaste at least twice a day
- floss at least once a day (one of the most beneficial things you can do to prevent disease in your oral cavity)
- have your teeth cleaned by a dental professional every six months
- avoid tobacco products
- follow a high-fiber, low-fat, low-sugar diet that includes plenty of fruits and vegetables
- limit sugary snacks and drinks

Foods with hidden sugars include:

- condiments such as ketchup and barbecue sauce
- sliced fruit or applesauce in cans or jars that have added sugars
- flavored yogurt
- pasta sauce
- sweetened iced tea
- soda
- sports drinks
- juice or juice blends
- granola and cereal bars
- muffins

Get more tips on preventing oral health problems. Good oral health is especially important to groups such as children, pregnant women, and older adults. What you should know about your child's oral health The American Academy of Pediatrics (AAP) recommends that children start seeing a dentist by their first birthday.

Children are highly susceptible to dental cavities and tooth decay, especially those who bottle feed. Cavities may be caused by too much sugar left on the teeth after bottle feeding.

To avoid baby bottle tooth decay, you should do the following:

- only bottle feed during meal times
- wean your child off of a bottle by the time they're one year old
- fill the bottle with water if you must give them a bottle at bedtime
- begin brushing with a soft baby toothbrush once their baby teeth start to come in; you should use only water until your child learns not to swallow the toothpaste
- start seeing a pediatric dentist regularly for your child
- ask your child's dentist about dental sealants

Baby bottle tooth decay is also known as early childhood caries (ECC). Go [here](#) to find out more ways you can prevent ECC.

**What men need to know about oral health** According to the American Academy of Periodontology, men are less likely to take good care of their teeth and gums than women. Compared to women, men are less likely to brush twice per day, floss regularly, and seek preventive dental care. Oral and throat cancer is more common in men. A 2008 study showed that men with a history of periodontal disease are 14 percent more likely to develop other types of cancer than men with healthy gums. It's important that men recognize the consequences of poor oral health and take action early in life.

**What women need to know about oral health** Due to changing hormones at various stages of their lives, women are at risk for several oral health issues.

When a woman first starts menstruating, she may experience mouth sores or swollen gums during her periods.

During pregnancy, increased hormones can affect the amount of saliva produced by the mouth. Frequent vomiting caused by morning sickness can result in tooth decay. You can receive dental care during pregnancy, but you should let your dentist know if you're pregnant.

During menopause, lower amounts of estrogen can increase your risk of gum disease. Some women may also experience a condition called burning mouth syndrome (BMS) during menopause. Learn about the different dental issues that women face throughout their lives. **What people with diabetes need to know about oral health** Diabetes affects the body's ability to fight off bacteria. This means that people with diabetes have a higher risk of having oral infections, gum disease, and periodontitis. They're at an increased risk of an oral fungal infection called thrush.

For people with diabetes to take charge of their oral health, they'll need to maintain control over their blood sugar levels. This is on top of brushing, flossing, and dentist's visits. Explore the link between type 2 diabetes and oral health. The bottom line about dental and oral health Your oral health has an effect on more than just your teeth. Poor oral and dental health can contribute to issues with your self-esteem, speech, or nutrition. They can also affect your comfort and overall quality of life. Many dental and oral problems develop without any symptoms. Seeing a dentist regularly for a checkup and exam is the best way to catch a problem before it gets worse.

Ultimately, your long-term outcome depends on your own efforts. You can't always prevent every cavity, but you can reduce your risk of severe gum disease and tooth loss by staying on top of your daily oral care.

49. The anerobic bacteria on the surface of the tongue have no use and can be effectively destroyed by daily use of an antimicrobial mouthwash.

- a. True
- b. False

Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans Anni Vanhataloa,\*, Jamie R. Blackwella, Joanna E. L'Heureuxa, David W. Williamsb, Ann Smithc, Mark van der Giezena, Paul G. Winyardd, James Kellya, Andrew M. Jonesa aCollege of Life and Environmental Sciences, University of Exeter, Exeter EX1 1TE, UK bSchool of Dentistry, Cardiff University, Cardiff CF14 4XY UK cDivision of Population Medicine, Cardiff University, Cardiff CF14 4XY UK dUniversity of Exeter Medical School, University of Exeter, Exeter EX1 1TE, UK

A R T I C L E I N F O

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ABSTRACT

Imbalances in the oral microbial community have been associated with reduced cardiovascular and metabolic health. A possible mechanism linking the oral microbiota to health is the nitrate (NO<sub>3</sub><sup>-</sup>)-nitrite (NO<sub>2</sub><sup>-</sup>)-nitric oxide (NO) pathway, which relies on oral bacteria to reduce NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup>. NO (generated from both NO<sub>2</sub><sup>-</sup> and Larginine) regulates vascular endothelial function and therefore blood pressure (BP). By sequencing bacterial 16S rRNA genes we examined the relationships between the oral microbiome and physiological indices of NO bioavailability and possible changes in these variables following 10 days of NO<sub>3</sub><sup>-</sup> (12mmol/d) and placebo supplementation in young (18–22yrs) and old (70–79yrs) normotensive humans (n=18). NO<sub>3</sub><sup>-</sup> supplementation altered the salivary microbiome compared to placebo by increasing the relative abundance of Proteobacteria (+225%) and decreasing the relative abundance of

Bacteroidetes (−46%;  $P < 0.05$ ). After NO<sub>3</sub>-supplementation the relative abundances of Rothia (+127%) and Neisseria (+351%) were greater, and Prevotella (−60%) and Veillonella (−65%) were lower than in the placebo condition (all  $P < 0.05$ ).

NO<sub>3</sub>-supplementation increased plasma concentration of NO<sub>2</sub>- and reduced systemic blood pressure in old (70–79yrs), but not young (18–22yrs), participants. High abundances of Rothia and Neisseria and low abundances of Prevotella and Veillonella were correlated with greater increases in plasma [NO<sub>2</sub>-] in response to NO<sub>3</sub>-supplementation. The current findings indicate that the oral microbiome is malleable to change with increased dietary intake of inorganic NO<sub>3</sub>-, and that diet-induced changes in the oral microbial community are related to indices of NO homeostasis and vascular health in vivo.

### 1. Introduction

Inorganic nitrate (NO<sub>3</sub>-) is a natural part of the human diet that is found in high concentrations in many vegetables. NO<sub>3</sub>- itself is biologically inert and human cells are believed to lack NO<sub>3</sub>--reductase capability. However, commensal bacteria in the oral cavity can use nitrate as a terminal electron acceptor for ATP synthesis, reducing NO<sub>3</sub>- to nitrite (NO<sub>2</sub>-; which can be vasoactive in low-oxygen and low-pH conditions) and this NO<sub>2</sub>- can be further reduced to the potent vasodilator, nitric oxide (NO) [9]. The NO<sub>3</sub>--NO<sub>2</sub>--NO reduction pathway underpins the discovery that dietary NO<sub>3</sub>- supplementation through consumption of NO<sub>3</sub>- salts [27] or vegetable products such as beetroot juice [23,40] reduces blood pressure (BP) in healthy young and old humans. The importance of a functional oral microbiome for the NO<sub>3</sub>--NO<sub>2</sub>-

NO reduction pathway is highlighted in cases where use of antibacterial mouthwash markedly blunts the increase in plasma and saliva NO<sub>2</sub> concentrations and associated decrease in BP following ingestion of a standardised NO<sub>3</sub>- dose [14,22,31]. Epidemiological studies also indicate that dysbiosis of the oral microbial community is associated with poor cardiovascular health [5]. Conversely, a diet rich in vegetables, which contain high concentrations of inorganic NO<sub>3</sub>-, significantly protects against both coronary heart disease and stroke [16,2,20]. Ageing has been associated with reduced salivary flow rate ('dry mouth') and altered oral bacterial colonisation [33,42], but it is not known whether the abundances of NO<sub>3</sub>- reducing oral bacteria decline with age. Dietary NO<sub>3</sub>- intake, and the abundance of NO<sub>3</sub>--reducing oral bacteria, therefore represent routes to lower blood pressure and maintain and improve cardiovascular health across the human lifespan. It is possible that dietary NO<sub>3</sub>- as a prebiotic treatment might

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promote proliferation of NO<sub>3</sub>- reducing bacteria. In a rodent model, a NO<sub>3</sub>--rich diet over 7 days increased abundance of oral bacteria (Streptococcus and Haemophilus) that contain NO<sub>3</sub>- reductase genes [17]. In saliva samples of hypercholesterolaemic humans, 6 weeks of NO<sub>3</sub>-supplementation with beetroot juice significantly increased the abundance of Neisseria flavescens and tended to increase Rothia mucilaginosa which are known NO<sub>3</sub>- reducers

[39]. These studies indicate that increased dietary NO<sub>3</sub>- intake may alter the oral microbiome in a way which enhances an individual's ability to reduce ingested NO<sub>3</sub>-, resulting in greater plasma NO<sub>2</sub>- concentration and a greater reduction in systemic blood pressure. However, characterisation of potential changes in the oral microbiome of healthy young and old humans in response to NO<sub>3</sub>- supplementation is lacking. In the present study, we used 16S rRNA gene sequencing to investigate whether abundances of NO<sub>3</sub>--reducing bacteria on the surface of the human tongue modulate an individual's response to NO<sub>3</sub>- supplementation in young (18–22 years) and old (70–79 years) normotensive adults. We hypothesised that at baseline, abundances of known NO<sub>3</sub>--reducing bacteria (including *Neisseria*, *Prevotella*, *Rothia*, *Veillonella* and *Actinomycetales*) would be greater in young compared to old participants, and that high abundances of these bacteria at baseline would be associated with higher plasma NO<sub>2</sub>- concentrations, and greater changes in blood pressure and arterial stiffness in response to NO<sub>3</sub>- supplementation. We also investigated whether 10 days of regular dietary NO<sub>3</sub>- ingestion altered the oral microbiome compared with placebo supplementation. It was hypothesised that oral microbiomes would be different between placebo and NO<sub>3</sub>- conditions, and specifically that the relative abundances of bacteria capable of NO<sub>3</sub>reduction would be greater after NO<sub>3</sub>- compared to placebo supplementation.

## 2. Methods

### 2.1. Ethical approval

The study was approved by the institutional Ethics Committee (Sport and Health Sciences, University of Exeter) and conducted in accordance with the code of the ethical principles of the World Medical Association (Declaration of Helsinki). All participants gave their written, informed consent before the commencement of the study, once the experimental procedures, associated risks, and potential benefits of participation had been explained.

### 2.2. Study participants

Nine old adults including six females (mean ± SD, age 75 ± 3yrs, age range 70–79yrs, height 162 ± 6cm, body mass 61.8 ± 14.0kg) and three males (age 73 ± 5yrs, age range 70–78yrs, height 172 ± 4cm, body mass 77.7 ± 11.6kg) and nine young adults including five females (age 20 ± 1yrs, age range 19–22yrs, height 168 ± 7cm, body mass 67.9 ± 10.3kg) and four males (age 20 ± 2yrs, age range 18–22yrs, height 180 ± 4cm, body mass 73.4 ± 12.9kg) volunteered to participate in this study (Table 1). All participants were of Caucasian ethnicity. The nine old adults represented a subsample of a larger cohort tested for a Dunhill Medical Trust funded project (R269/1112) from which the microbiome of the tongue and saliva were retrospectively analysed. Participants were screened prior to participation to ensure suitability for the study. All participants were ostensibly healthy and were not taking medication or dietary supplements. None of the participants were tobacco smokers and all reported having no oral diseases. Participants were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3h postprandial, and to avoid strenuous physical exertion in the 24h preceding each laboratory visit. Participants were also asked to refrain from caffeine and alcohol intake 6 and 24h before each test, respectively. All tests were performed at approximately the same time of day ( ± 2h) for each participant.

### 2.3. Experimental design

Prior to commencing dietary supplementation, participants visited the laboratory for health screening and familiarisation to test protocols. Participants completed a salivary

flowratequestionnaire(SFR-Q;[11]). The SFR-Q included eleven questions which asked the participant to rate the frequency of various symptoms of low salivary flow rate on a scale of 1 ('never') to 5 ('very often'). Participants then underwent two 10-day dietary supplementation periods with NO<sub>3</sub><sup>-</sup> and placebo in a randomised, double-blind, cross-over design (Fig. 1). On days 8, 9 and 10 of each supplementation period, participants returned to the laboratory. Upon arrival at the laboratory on days 8, 9 and 10 venous blood samples were collected for the measurement of plasma [NO<sub>2</sub><sup>-</sup>] and [NO<sub>3</sub><sup>-</sup>] and resting BP was measured. The mean values of three measurements of [NO<sub>2</sub><sup>-</sup>], [NO<sub>3</sub><sup>-</sup>] and BP were used for further analyses. Saliva samples were also collected on each visit and these three samples were pooled for analysis of the salivary microbiome. Arterial stiffness was assessed on one occasion (day 8, 9 or 10) using radial-femoral pulse wave velocity (PWV).

#### 2.4. Supplementation

The supplements were NO<sub>3</sub><sup>-</sup>-rich concentrated beetroot juice (BR)

Table 1 Participant characteristics, nitrate (NO<sub>3</sub><sup>-</sup>) dose and plasma [NO<sub>2</sub><sup>-</sup>] responsiveness to supplementation, and salivary flow rate questionnaire (SFR-Q) results. The young and old participants were similar in terms of body mass and BMI. The NO<sub>3</sub><sup>-</sup> dose was similar in both groups but old participants had a greater increase than the young in plasma [NO<sub>2</sub><sup>-</sup>] in response to supplementation. The young reported feeling more frequent symptoms of low salivary flow rate than the old participants.

Sex Age (yrs) Body mass (kg)

BMI (kg/ m<sup>2</sup>)

NO<sub>3</sub><sup>-</sup> dose (mmol/ kg/d)

Δ[NO<sub>2</sub><sup>-</sup>]/ NO<sub>3</sub><sup>-</sup> dose (nM/mmol/ kg/d)

SFR-Q mean score

OLD	1 F	77	88.0	33.1	0.14	7543	1.2	2 F	79	66.2	22.1	0.19	2325	1.3	3 F	70	60.0	24.3	0.21		
	2962	1.7	4 F	76	53.1	20.7	0.23	3942	1.1	5 F	72	51.4	20.1	0.24	4819	1.7	6 F	74	52.3	20.2	
		0.24	5428	1.9	7 M	78	88.8	31.1	0.14	812	2.0	8 M	70	78.6	25.4	0.16	5461	1.0	9 M	70	65.6
		22.7	0.19	4829	1.5	Mean	74.0	67.1	24.4	0.19	4236	1.4	SD	3.6	14.8	4.7	0.04	1988	0.4	YOUNG	
	10 F	22	71.5	29.4	0.17	803	1.9	11 F	19	60.8	22.9	0.20	2202	2.5	12 F	19	64.5	22.6	0.19	880	
		1.6	13 F	20	69.7	24.4	0.18	1604	2.7	14 F	19	85.2	28.5	0.15	999	1.5	15 M	18	55.7	18.0	0.22
		2371	1.5	16 M	22	81.2	23.7	0.15	3254	1.6	17 M	19	72.3	22.3	0.17	1127	2.1	18 M	19	84.4	
		26.0	0.15	70	2.1	Mean	19.7*	71.7	24.2	0.18	1479*	2.0*	SD	1.4	10.4	3.5	0.03	977	0.4		
	OVERALL (OLD + YOUNG)	Mean	46.8	69.4	24.3	0.18	2857	1.7	SD	28.1	12.6	4.0	0.03	2079	0.5						

F, female; M, male; BMI, body mass index; Δ[NO<sub>2</sub><sup>-</sup>]/NO<sub>3</sub><sup>-</sup> dose, change in plasma [NO<sub>2</sub><sup>-</sup>] relative to dose of NO<sub>3</sub><sup>-</sup> ingested per kg body mass; SFR-Q, salivary flow rate questionnaire.

\* Different from old, P < 0.05.

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(2× 70ml d<sup>-1</sup>, each 70ml containing ~ 6.2mmol NO<sub>3</sub><sup>-</sup>; Beet It, James White Drinks, Ipswich, UK) and NO<sub>3</sub><sup>-</sup>-depleted concentrated beetroot juice placebo (PL) (2× 70ml d<sup>-1</sup>, each 70ml containing ~ 0.01mmol NO<sub>3</sub><sup>-</sup>; Beet It, James White Drinks, Ipswich, UK). The PL was indistinguishable from the BR supplement in appearance, taste and smell. Participants were instructed to consume one 70-ml beverage in the morning and one in the afternoon. On testing days, participants were asked to ingest one 70-ml beverage in the morning and one 2.5h prior to their laboratory visit. A washout period of at least three days and up to 47

days ( $18 \pm 14$  days) separated the supplementation periods. Participants were instructed to maintain their normal daily activities, food intake and oral hygiene regime throughout the study. However, participants were instructed to refrain from using antibacterial mouthwash during the study period. Participants were advised that supplementation may cause beeturia (red urine) and red stools temporarily, but that such side effects were harmless.

### 2.5. Oral bacteria

Oral swabs of the tongue dorsum were collected at baseline. Saliva samples (~ 1ml) were collected by expectoration, without stimulation, over a period of 5min on three occasions following PL and BR supplementation periods. Oral swab and saliva samples were stored at  $-80^{\circ}\text{C}$  until analysis. Genomic DNA was isolated from tongue swabs using a Genra Puregene Buccal Cell Kit (Qiagen, Germantown, MD), and from saliva samples following the methods of Goode et al. [13]. Double-stranded DNA concentration was fluorometrically quantified (Qubit 3.0 high-sensitivity fluorescence detection, ThermoFisher Scientific, Waltham, MA). Library preparation employed a NEXTflex 16S V1-V3 Amplicon-Seq Kit (Bioo Scientific, Austin, USA). The 16S V1-V3 rDNA region was amplified using 5ng of dsDNA and subjected to 8 thermal cycles of 30s at  $98^{\circ}$ , 30s at  $60^{\circ}$  and 30s at  $72^{\circ}$  with primers A and B (Table S1). Following AMPure® XP bead cleanup (Becton Dickinson, Franklin Lakes, NJ), a subsequent PCR with indexing primers to identify individual samples, containing Illumina flow cell binding sites, was performed. The samples were sequenced using paired-end 300 base pair (bp) MiSeq Illumina platform (Illumina, San Diego, CA) using v3 MiSeq reagents. For each sample, the nucleotide sequence data in FASTQ format was trimmed using Trim-Galore! (Krueger F. Trim-Galore!, accessible at [http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)). Quality trimming was performed by removing low-quality bases from the 3' read ends. The adapter sequences were subsequently removed from the 3' end (the first 13 base pairs). Trim-Galore! paired-end validation was performed to remove short sequences once the trimming was complete, where the minimum specified length was 20bp. The bacterial taxonomies and abundance were assigned using Kraken standard build, which uses the genomes in Refseq and NCBI taxonomic information (Kraken manual, accessible at: <http://ccb.jhu.edu/software/kraken/MANUAL.html#kraken-databases>). The paired read sequences were classified and processed by the Kraken Taxonomic Sequence Classification System [41]. Variations in the V1-V3 regions enabled NCBI taxonomic identification, and kraken-translate was used to translate the NCBI Identifiers to taxonomy identifiers. A Kraken report was generated for each sample, which was visualised using Krona bioinformatics pie charts [32].

### 2.6. Plasma [NO<sub>3</sub>-] and [NO<sub>2</sub>-]

Blood samples for determination of plasma [NO<sub>2</sub>-] and [NO<sub>3</sub>-] were collected from an antecubital vein into lithium heparin tubes and centrifuged for 8min at 3000g and  $4^{\circ}\text{C}$  within 2min of collection. Plasma was extracted and samples stored at  $-80^{\circ}\text{C}$  for later determination of [NO<sub>3</sub>-] and [NO<sub>2</sub>-] using a modified chemiluminescence technique as previously described [23].

### 2.7. Blood pressure and arterial stiffness

Blood pressure of the brachial artery was measured following 10min of seated rest in a quiet room using an automated sphygmomanometer (Dinamap Pro, GE Medical Systems, Tampa, USA). A total of four measurements were taken, with the mean of the final three

measurements recorded. The mean of the systolic (SBP), diastolic (DBP) and mean arterial pressure (MAP) measurements made over three laboratory visits in each condition were calculated for each individual and used for subsequent analyses. Arterial stiffness was estimated via pulse-wave velocity (PWV) (Complior SP; Alam Medical, Vincennes, Paris, France). Electrodes were placed on the carotid, femoral, and radial arteries, and the pulse transit time was calculated and recorded. A mean of three measurements was calculated and used for subsequent analyses. The position of each electrode was measured in relation to the nearest bony landmark to enable precise reproduction of the position of the electrodes in each condition.

### 2.8. Statistical analyses

The Kraken raw data output of phylogenetic data were analysed using R-script (R Development Core Team 2008), SPSS V20 and Microsoft Excel. Non-metric multidimensional scaling (NMDS) was used to assess the level of microbiome similarity between young and old, and PL and BR conditions using non-parametric relationships, and analysed using ADONIS (Vegan R Software). Differences between PL and BR conditions were assessed using paired t-tests with Bonferroni-Hochberg correction on bacteria that made up > 0.01% of bacteria (R statistical software). The Shannon-Wiener diversity index ( $H'$ ) was used to explore differences in diversity (Vegan R Software). Paired-samples t tests were used to assess differences between BR and PL conditions in plasma [NO<sub>2</sub>-] and [NO<sub>3</sub>-], BP and arterial stiffness. Relationships between plasma NO biomarkers, oral microbiome and physiological responses to supplementation were assessed using Pearson's correlation coefficients. Statistical significance was accepted when  $P < 0.05$  and statistical trend was defined as  $P < 0.10$ . Data were expressed as mean  $\pm$  SD.

### 3. Results

The young and old participants were similar in terms of body mass and BMI (Table 1). The young participants had a greater mean score in SFR-Q than the old participants (Table 1), indicative of more frequent self-reported symptoms of low salivary flow rate. The young Fig. 1. Participants underwent 10-day supplementation periods with nitrate (~12.4 mmol/d) and placebo in a balanced cross-over design. Screening, protocol familiarisation and Salivary Flow Rate Questionnaires (SFR-Q) were completed at baseline. Measurements of plasma nitrite (NO<sub>2</sub>-) and nitrate (NO<sub>3</sub>-) concentrations, blood pressure (BP) of the brachial artery, arterial stiffness as carotid-femoral pulse wave velocity (PWV), and the collection of saliva samples for microbiome analysis were undertaken on days 8, 9 and 10 of each supplementation period.

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participants reported greater frequency of sensations associated with dry mouth (old  $1.6 \pm 1.0$ , young  $2.7 \pm 0.5$ ;  $P < 0.05$ ) and having difficulty eating dry foods (old  $1.1 \pm 0.3$ , young  $1.8 \pm 0.4$ ;  $P < 0.05$ ).

#### 3.1. NO biomarkers, blood pressure and arterial stiffness

The NO<sub>3</sub>- dose relative to body mass was not different between young and old participants, but the latter had a greater increase in plasma [NO<sub>2</sub>-] in response to BR supplementation (Table 1). Plasma [NO<sub>3</sub>-] and [NO<sub>2</sub>-] were significantly higher following BR supplementation compared with PL (all  $P < 0.05$ ; Fig. 2, panels A and B). BR supplementation increased plasma [NO<sub>3</sub>-] relative to PL by a similar amount in old ( $1509 \pm 744\%$ ) and young participants ( $1481 \pm 909\%$ ) (Fig. 2C), but the increase in [NO<sub>2</sub>-] was

greater in old ( $648 \pm 477\%$ ) compared to young participants ( $365 \pm 249\%$ ,  $P < 0.05$ ; Fig. 2D). There were no differences between young and old participants in plasma  $[\text{NO}_3^-]$  in PL (old:  $28 \pm 12\mu\text{M}$ , young:  $28 \pm 14\mu\text{M}$ ;  $P > 0.05$ ) or BR conditions (old:  $379 \pm 55\mu\text{M}$ , young:  $366 \pm 101\mu\text{M}$ ;  $P > 0.05$ ). Plasma  $[\text{NO}_2^-]$  tended to be greater in the old in PL (old:  $173 \pm 97\text{nM}$ , young:  $104 \pm 63\text{nM}$ ;  $P=0.09$ ) and was significantly greater in the old than young participants in the BR condition (old:  $1029 \pm 393\text{nM}$ , young:  $380 \pm 175\text{nM}$ ;  $P < 0.05$ ). MAP, SPB, DBP and PWV were not different between PL and BR conditions across all participants ( $n=18$ ,  $P > 0.05$ ; Fig. 3, panels A, D, G and J). In old participants ( $n=9$ ), SBP and MAP were significantly lower following BR supplementation compared with PL (Fig. 3, panels B and E). Changes between PL and BR ( $\Delta$ ) in MAP, SBP, and DBP between PL and BR conditions inversely correlated with the change in plasma  $[\text{NO}_2^-]$  relative to  $\text{NO}_3^-$  dose per kg body mass ( $\Delta[\text{NO}_2^-]/\text{NO}_3^-$  dose; Fig. 3, panels C, F and I). BR supplementation did not significantly alter radial-femoral PWV (Fig. 3, panels J and K) across all participants, but  $\Delta$ PWV between BR and PL in the old participants (increase of  $4.3 \pm 10.9\text{ms}^{-1}$ ;  $n=9$ ) was different ( $P < 0.05$ ) from  $\Delta$ PWV in the young participants (decrease of  $-6.8 \pm 9.8\text{ms}^{-1}$ ;  $n=9$ ).  $\Delta$ PWV positively correlated with  $\Delta[\text{NO}_2^-]/\text{NO}_3^-$  dose (Fig. 3L). Absolute plasma  $[\text{NO}_2^-]$  or  $[\text{NO}_3^-]$  measured in the PL condition were not correlated with  $\Delta[\text{NO}_2^-]$ ,  $\Delta$ DBP,  $\Delta$ SBP,  $\Delta$ MAP or  $\Delta$  PWV. One old male participant did not wish to undertake PWV measurement and therefore all PWV data are derived from 17 participants.

### 3.2. Tongue microbiome at baseline

Relative abundances of the five main oral bacterial phyla in tongue swab samples collected at baseline were Bacteroidetes  $32 \pm 9\%$ , Fusobacteriales  $27 \pm 11\%$ , Proteobacteria  $20 \pm 7\%$ , Firmicutes  $17 \pm 5\%$  and Actinobacteria  $1 \pm 1\%$ . The most abundant bacterial species found on the tongue were *Fusobacterium nucleatum* subsp. *nucleatum* ( $16 \pm 8\%$ ), *Prevotella melaninogenica* ( $14 \pm 7\%$ ), *Campylobacter concisus* ( $13 \pm 8\%$ ), *Leptorichia buccalis*, ( $7 \pm 6\%$ ) *Veillonella parvula* ( $5 \pm 3\%$ ), *Prevotella intermedia* ( $4 \pm 2\%$ ), *Fusobacterium nucleatum* subsp. *vincentii* ( $3 \pm 3\%$ ) and *Neisseria meningitidis* ( $3 \pm 3\%$ ). Correlations between relative abundances of selected taxonomic units of tongue bacteria and physiological responses to BR supplementation are shown in Table 2. The greatest decreases in BP were associated with high abundances at baseline of *Fusobacterium nucleatum* subsp. *vincentii* and *nucleatum*, and order Actinomycetales, whereas a high relative abundance of *Prevotella melaninogenica* was associated with a greater mean score in SFR-Q, and smaller changes in plasma  $[\text{NO}_2^-]$ , SBP and PWV in response to BR supplementation (Table 2).

### 3.3. Saliva microbiome after PL and BR supplementation

Relative abundances of the main phyla of oral bacteria differed between PL and BR conditions (Fig. 4). Relative abundance of Proteobacteria was greater and Bacteroidetes was lower following BR compared with PL ( $P < 0.05$ ), while abundances of Firmicutes and Fig. 2. Plasma  $[\text{NO}_3^-]$  (panel A) and  $[\text{NO}_2^-]$  (panel B) were significantly greater after nitrate supplementation (white bars) compared to placebo (black bars) ( $n=18$ ). The change ( $\Delta$ ) in plasma  $[\text{NO}_3^-]$  between nitrate and placebo conditions was similar in young ( $n=9$ ) and old participants ( $n=9$ ) (panel C), but  $\Delta[\text{NO}_2^-]$  was significantly greater in the old compared to young participants (panel D). Error bars indicate standard deviations and black squares (panels C and D) indicate means for young and old participants. \* $P < 0.05$ .

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Fusobacteria tended to be lower following BR supplementation compared with PL ( $P < 0.10$ ). NMDS plots revealed that oral microbial communities differed significantly between PL and BR supplemented conditions (Fig. 5A), but there were no differences between young and old participants (Fig. 5B). A Shannon diversity index revealed no significant differences in species diversity between PL and BR conditions (Fig. 6). Overall, 52 taxonomic units were significantly different between BR and PL conditions. Fig. 7 illustrates statistically significant differences at genera and species levels between PL and BR. The trend for reduction in the relative abundance of Firmicutes following BR was primarily due to a decrease in Veillonella ( $-65\%$ ), including a 65% decrease in Veillonella parvula species (both  $P < 0.05$ ), while the order Lactobacilliales and genus Streptococcus were not affected by BR ( $P > 0.05$ ). Within the phylum Bacteroidetes, BR resulted in a reduction in the genus Prevotella ( $-60\%$ ), and specifically *P. melaninogenica* ( $-67\%$ ) compared to PL (both  $P < 0.05$ ). The increase in Proteobacteria after BR stemmed from an increase in the order Neisseriales ( $+348\%$ ), containing the genus Neisseria ( $+351\%$ ) and *N. meningitidis* ( $+439\%$ ) (all  $P < 0.05$ ), while there were no statistically significant changes in the genera Campylobacter or Haemophilus. Proportions of the

Fig. 3. Meanarterial pressure (MAP; panel A), systolic blood pressure (SBP; panel D), diastolic blood pressure (DBP; panel G) and pulse wave velocity (PWV; panel J) were not different between placebo and nitrate conditions across all participants ( $n=18$ ). The old participants ( $n=9$ ) showed greater reductions ( $\Delta$ ) in MAP, SBP and DBP between placebo and nitrate conditions than the young participants ( $n=9$ ; panels B, E and H), as well as a greater increase in PWV (young  $n=9$ , old  $n=8$ ; panel K).  $\Delta$ MAP,  $\Delta$ SBP and  $\Delta$ DBP inversely correlated with the change in plasma  $[\text{NO}_2^-]$  relative to nitrate dose ( $\Delta[\text{NO}_2^-]/\text{NO}_3^-$  dose) (panels C, F and I) and  $\Delta$ PWV positively correlated with  $\Delta[\text{NO}_2^-]/\text{NO}_3^-$  dose (panel L). \* $P < 0.05$ .

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phylum Actinobacteria were not significantly different between PL and BR, but there was an increase in the genus Rothia ( $+127\%$ ,  $P < 0.05$ ) and *R. mucilaginosa* ( $+234\%$ ,  $P < 0.05$ ) after BR supplementation relative to PL. There was insufficient microbial DNA in a saliva sample of one young male participant and therefore the saliva microbiome data were for 17 participants. Correlations across PL and BR conditions showed that plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  positively correlated with relative abundances of Rothia and Neisseria and inversely correlated with Prevotella and Veillonella (Table 3). PWV positively correlated with relative abundance of Rothia and *R. mucilaginosa* (Table 3).

#### 4. Discussion

We used an in vivo experimental model and bacterial 16S rRNA gene sequencing to examine relationships between the oral microbiome and physiological indices of NO bioavailability in humans and changes in these variables following  $\text{NO}_3^-$  supplementation. The principal finding of this study was that dietary  $\text{NO}_3^-$  supplementation altered the salivary microbiome in young ( $\sim 20$  yrs) and old ( $\sim 74$  yrs) normotensive humans, such that it increased relative abundances of some bacteria capable of  $\text{NO}_3^-$  reduction (Rothia and Neisseria) while reducing the abundances of other  $\text{NO}_3^-$  reducers (Prevotella and Veillonella).  $\text{NO}_3^-$

supplementation increased NO bioavailability in all participants, as indicated by plasma concentrations of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>, and reduced systemic blood pressure in the old, but not young, participants. Across placebo and NO<sub>3</sub><sup>-</sup> supplemented conditions, high abundances of *Rothia* and *Neisseria* and low abundances of *Prevotella* and *Veillonella* were associated with high NO bioavailability. The current findings indicate that the oral microbial community was malleable to change with increased dietary intake of inorganic NO<sub>3</sub><sup>-</sup>, and, importantly, that the oral microbiome was related to indices of NO homeostasis and vascular health in vivo.

#### 4.1. Relationships between the tongue microbiome at baseline and responsiveness to NO<sub>3</sub><sup>-</sup> supplementation

It has been proposed that the oral microbiome modulates the magnitude of plasma [NO<sub>2</sub><sup>-</sup>] increase, as well as changes in associated physiological indices, in response to NO<sub>3</sub><sup>-</sup> supplementation [6,17,18,25]. A recent study indicated that a composite relative abundance of seven species (including *Prevotella melaninogenica*, *Veillonella parvula*, and *Rothia mucilaginosa*) was positively correlated with the rise in salivary [NO<sub>2</sub><sup>-</sup>], but not plasma [NO<sub>2</sub><sup>-</sup>], in response to acute ingestion of a single NO<sub>3</sub><sup>-</sup> bolus [7]. We found that individuals who had high proportions of *P. melaninogenica* and *Campylobacter concisus* at

Table 2 Correlation coefficients for relationships between selected taxonomic units of the tongue microbiome (% of total bacteria) at baseline and subsequent changes between placebo and NO<sub>3</sub><sup>-</sup> supplementation in plasma [NO<sub>2</sub><sup>-</sup>], blood pressure and arterial stiffness. PWV data were not available for one old male participant, such that ΔPWV correlations are for n=17. Δ[NO<sub>2</sub><sup>-</sup>]/NO<sub>3</sub><sup>-</sup> dose (nM/mmol/ kg/D) ΔDBP (mmHg) ΔSBP (mmHg) ΔMAP (mmHg) ΔPWV (m/s) SFR-Q mean score

Δ[NO <sub>2</sub> <sup>-</sup> ]/NO <sub>3</sub> <sup>-</sup> dose	-0.57*	-0.73**	-0.65**	0.65**	-0.52*	Actinobacteria	Actinomycetales	(order)	0.40	-0.39	-0.46#	-0.46#	0.47#	-0.004	Micrococcales	(order)	-0.41	0.25	0.36	0.40				
	-0.21	-0.04	<i>Rothia</i> (genus)	-0.20	0.10	0.32	0.37	-0.07	0.20	<i>Rothia mucilaginosa</i>	-0.22	0.17	0.38	0.44#	-0.08	0.18	<i>Proteobacteria</i>	<i>Neisseria</i> (genus)	0.21	-0.06	0.06	0.29	0.59*	-0.39
	-0.09	-0.08	0.044	0.36	0.13	-0.19	<i>Campylobacter concisus</i>	-0.55*	0.43#	0.65**	0.34	-0.62**	0.30	<i>Bacteroidetes</i>	<i>Prevotella</i> (genus)	-0.49*	0.19	0.43#	0.41#					
	-0.52*	0.51*	<i>Prevotella melaninogenica</i>	-0.57*	0.16	0.53*	0.37	-0.68**	0.49*	<i>Firmicutes</i>	<i>Veillonella</i> (genus)	-0.22	0.22	0.35	0.09	-0.02	0.46#	<i>Veillonella parvula</i>	-0.19	0.22	0.35			
	0.10	0.02	0.41#	<i>Fusobacteria</i>	<i>Fusobacterium nucleatum</i> subsp. <i>nucleatum</i>	0.44#	-0.17	-0.56*	-0.30	0.32	-0.29	<i>Fusobacterium nucleatum</i> subsp. <i>vincentii</i>	0.55*	-0.43#	-0.60**	-0.63**	0.45#	-0.36						

Δ=change between placebo and NO<sub>3</sub><sup>-</sup>; [NO<sub>2</sub><sup>-</sup>]/NO<sub>3</sub><sup>-</sup> dose =plasma [nitrite] relative to nitrate dose per kg body mass ingested; DBP=diastolic blood pressure; SBP=systolic blood pressure; MAP=mean arterial pressure; PWV=pulse wave velocity; SFR-Q=salivary flow rate questionnaire. \*\* P < 0.01. \* P < 0.05. # P < 0.10.

Fig. 4. The proportions of five main phyla of oral bacteria identified in the saliva samples following 10 days of placebo (PL) and NO<sub>3</sub><sup>-</sup> supplementation (BR). \*Difference between PL and BR (P < 0.05).

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baseline were less responsive to chronic NO<sub>3</sub><sup>-</sup> supplementation, i.e. had smaller increases in plasma [NO<sub>2</sub><sup>-</sup>] and smaller or no reductions in BP, than those individuals who had low abundances of *P. melaninogenica* and *C. concisus*. In addition, a high mean SFR-Q score at

baseline, which indicated more frequent self-reported symptoms of dry mouth, was associated with greater abundance of *Prevotella* and a smaller increase in plasma [NO<sub>2</sub>-] in response to NO<sub>3</sub>- supplementation. *Campylobacter concisus* is believed to express dissimilatory NO<sub>3</sub>- reduction to NH<sub>3</sub> and its main physiological function is NO<sub>2</sub>- reduction [36], while *P. melaninogenica* has been shown to encode NO<sub>2</sub>-, but not NO<sub>3</sub>-, reductase genes [18]. It may, therefore, be speculated that during subsequent NO<sub>3</sub>- supplementation both *C. concisus* and *P. melaninogenica*, which were dominant species in tongue swab samples at baseline, acted as net consumers of NO<sub>2</sub>- in the oral cavity. In contrast, high abundances of *Fusobacterium nucleatum* subspecies and Actinomycetales at baseline were associated with greater increases in plasma [NO<sub>2</sub>-] and greater reductions in blood pressure in response to NO<sub>3</sub>- supplementation (Table 2).

Actinomycetales are generally obligate anaerobes, and includes several species such as *Actinomyces odontolyticus* and *Actinomyces naeslundii* that have been identified as effective NO<sub>3</sub>- reducers [10]. *Fusobacterium nucleatum* can reduce NO<sub>2</sub>-, but does not possess NO<sub>3</sub>-reductase genes. However, *F. nucleatum* provides 'scaffolding' in biofilms, enabling microbial attachments [26], and it is possible that these bacteria may have facilitated attachment and proliferation of key NO<sub>3</sub>-reducing bacteria during dietary intervention.

#### 4.2. Changes in the salivary microbiome after NO<sub>3</sub>- supplementation

We showed that, relative to placebo, NO<sub>3</sub>- supplementation altered the proportions of the main oral microbial phyla by decreasing the Bacteroidetes and increasing the Proteobacteria. At the genus level, NO<sub>3</sub>- supplementation significantly increased relative abundances of the previously identified NO<sub>3</sub>- reducers *Neisseria* [18] and *Rothia* [10] in saliva. This was consistent with a report of a significant increase in *N. flavescens* and a trend for an increase in *R. mucilaginosa* in saliva samples of hypercholesterolaemic patients after 6 weeks of NO<sub>3</sub>- supplementation [39]. A novel finding in the present study was that a high abundance of the facultative anaerobe *R. mucilaginosa* was associated with faster pulse wave velocity, indicative of lower arterial stiffness. These data suggest that high relative abundances of bacteria belonging to *Neisseria* and *Rothia* were related to high NO bioavailability and may promote vascular health. We also found that compared to placebo, NO<sub>3</sub>- supplementation decreased relative abundances of the obligate anaerobic bacteria *Prevotella* and *Veillonella* in saliva. This finding appears to contradict studies that have used in vitro approaches to identify key oral NO<sub>3</sub>-reducing taxa. Hyde et al. [18] categorised biofilms prepared from tongue swab samples of six healthy humans as best, intermediate and worst NO<sub>3</sub>- reducers and found greater abundances of both *Prevotella* and *Veillonella* in the best versus worst NO<sub>3</sub>- reducing biofilms. Using tongue swab samples from ten healthy humans, which were incubated on solid medium under aerobic and anaerobic conditions and analysed by 16S rDNA sequencing, Doel et al. [10] concluded that *Veillonella* were the most prevalent oral NO<sub>3</sub>- reducers and were major contributors to net NO<sub>2</sub>- production. Intricate metabolic interactions among the oral microbiota might mean that increased NO<sub>3</sub>- availability in the oral cavity may not facilitate the growth of all bacteria capable of NO<sub>3</sub>- reduction. It is not directly apparent why NO<sub>3</sub>- supplementation resulted in a decline in *Veillonella*. One factor that may have contributed to proliferation of some taxa and inhibition of others is the oral pH, which is a powerful modulator of the oral microbial community. Beetroot juice supplementation has been shown to increase oral pH from 7.0 to 7.5 [15], and notably, a pH of 8 is optimal for NO<sub>3</sub>- reductase activity

[38]. Monitoring of salivary pH alongside alterations in the oral microbiome during NO<sub>3</sub>- supplementation should be undertaken in future studies to address the possible effects of pH. Given that many oral bacteria with ability to reduce NO<sub>3</sub>- are also capable of downstream metabolism of the produced NO<sub>2</sub>-, it is important to differentiate between NO<sub>3</sub>-reducing bacteria in general and NO<sub>2</sub>- accumulating bacteria specifically. NO<sub>3</sub>-reducing oral bacteria have been identified in vitro from human samples, including *Veillonella*, *Actinomyces*, *Rothia*, *Staphylococcus* and *Propionibacterium* [10]. More recently Hyde et al. [18] added *Neisseria*, *Haemophilus parainfluenzae*, *Prevotella* (including *P. melaninogenica*) and *Granulicatella* to the list of candidate species for most potent contributors to oral NO<sub>2</sub>- production. We showed that some of the oral bacteria that have been proposed as key NO<sub>2</sub>- accumulators on the basis of in vitro experiments, such as *Veillonella* and *Prevotella*, do not thrive under high NO<sub>3</sub>- availability in vivo. Indeed, we found no significant changes in relative abundances of *Actinomyces*, *Staphylococcus*, *Propionibacterium*, *Granulicatella* or *Haemophilus* after NO<sub>3</sub>- supplementation. In order to contribute significantly to the amount of NO<sub>2</sub>- that is swallowed from the oral cavity, Fig. 5. Overall salivary microbiome composition illustrated by non-metric multidimensional scaling (NMDS) analysis. The salivary microbiome composition was different between nitrate (BR) and placebo (PL) conditions ( $P < 0.05$ ; panel A) but not between young and old participants ( $P > 0.05$ ; panel B).

Fig. 6. The Shannon diversity index indicated no statistically significant difference in species diversity between nitrate (BR) and placebo (PL) conditions.

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the bacteria need to reduce NO<sub>3</sub>- at a faster rate than they reduce NO<sub>2</sub>-, or not undertake downstream metabolism of NO<sub>2</sub>- at all. Overall, to maximise NO bioavailability from a NO<sub>3</sub>-rich diet, the composition of the oral microbial community needs to be such that it contains a greater quantity of net NO<sub>2</sub>- accumulators than net NO<sub>2</sub>- consumers. Further research is needed to establish whether the observed microbiome changes following chronic NO<sub>3</sub>- supplementation in the present cohort,

are replicated in different populations and whether such changes are associated with an increased capacity for acute NO<sub>3</sub>- reduction in the oral cavity. The present data suggest that the chronic (10-day) NO<sub>3</sub>- supplementation serves to change the relative abundance of a few, but not all, NO<sub>3</sub>- reducing taxa and that these changes are correlated with beneficial changes in NO bioavailability and indices of cardiovascular

Fig. 7. The genera (panels A and B) and species (panels C and D) that comprised > 0.01% of all bacteria and showed significant differences ( $P < 0.05$ ) between nitrate (BR) and placebo (PL) conditions.

Table 3 Correlation coefficients ( $r$ ) for relationships between relative abundances of selected taxonomic units of saliva microbiome (% of total bacteria) and plasma nitrate ([NO<sub>3</sub>-]) and nitrite ([NO<sub>2</sub>-]); diastolic (DBP), systolic (SBP) and mean arterial (MAP) blood pressure; and pulse wave velocity (PWV) across placebo and nitrate conditions.

Microbiome data were not available for one young

males subject and PWV data were not available for one older males subject, such that [NO<sub>3</sub>-], [NO<sub>2</sub>-] and BP correlations are for  $n=34$  and PWV correlations are for  $n=32$ . [NO<sub>3</sub>-] (μM) [NO<sub>2</sub>-] (nM) DBP (mmHg) SBP (mmHg) MAP (mmHg) PWV (m/s)

Actinobacteria Actinomycetales (order) 0.25 0.09 -0.01 -0.09 -0.05 0.06 Micrococcales (order) 0.37\* 0.23 -0.03 0.02 0.00 0.48\*\* Rothia (genus) 0.45\*\* 0.30# 0.04 0.03 0.04 0.45\* Rothia mucilaginosa 0.46\*\* 0.37\* 0.06 -0.03 -0.01 0.41\* Proteobacteria Neisseria (genus) 0.61\*\* 0.64\*\* 0.02 0.15 0.14 -0.13 Neisseria meningitidis 0.54\*\* 0.53\*\* -0.00 0.07 0.08 -0.23 Campylobacter concisus -0.26 -0.17 -0.17 0.08 0.02 0.42\* Bacteroidetes Prevotella (genus) -0.47\*\* -0.35\* 0.11 0.21 0.18 -0.18 Prevotella melaninogenica -0.47\*\* -0.35\* 0.06 0.17 0.12 -0.09 Firmicutes Veillonella (genus) -0.62\*\* -0.50\*\* -0.10 -0.01 -0.22 -0.07 Veillonella parvula -0.60\*\* -0.49\*\* -0.10 -0.03 -0.23 -0.08 Fusobacteria Fusobacterium nucleatum subsp. nucleatum -0.28 -0.18 0.00 0.08 0.05 -0.10 Fusobacterium nucleatum subsp. vincentii -0.16 -0.01 -0.03 0.12 0.07 -0.17

DBP=diastolic blood pressure; SBP=systolic blood pressure; MAP=mean arterial pressure; PWV=pulse wave velocity. \*\* P < 0.01. \* P < 0.05. # P < 0.10.

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health. It should be noted that elevated NO bioavailability may have further beneficial effects on aspects of healthy ageing, including maintenance of a strong immune response. A recent study showed that tongue microbiomes that had high abundances of Prevotella and Veillonella species were associated with elevated risks of all-cause mortality and mortality from pneumonia in frail elderly nursing home residents [21]. NO<sub>3</sub><sup>-</sup> supplementation in older people, which reduces the relative abundances of Prevotella and Veillonella, may therefore have potential to enhance the NO-mediated immune response in this high risk population.

#### 4.3. Differences in [NO<sub>2</sub><sup>-</sup>] and blood pressure responses between young and old participants

Previous studies have shown appreciable inter-individual variability in the plasma [NO<sub>2</sub><sup>-</sup>] and blood pressure responses to NO<sub>3</sub><sup>-</sup> ingestion in both young and older populations (e.g. [8,19,23]). We found that despite ingesting the same dose of NO<sub>3</sub><sup>-</sup> the old adults showed a greater plasma [NO<sub>2</sub><sup>-</sup>] increase than the young adults, and also exhibited a reduction in blood pressure which was absent in the young participants. The magnitude of the BP response to NO<sub>3</sub><sup>-</sup> supplementation is correlated with baseline blood pressure [22,40], such that in the present study the scope for a BP decrease in the young adults, who had a BP of ~ 112/ 63 mmHg in placebo, was likely small. Although the balance of evidence indicates that NO<sub>3</sub><sup>-</sup> represents an potent dietary means for reducing systemic BP [22,25,27,40,6], it is important to note that there are several studies that concur with the present finding of not showing a significant change in resting systolic or diastolic BP (or both) in young, healthy adults (e.g. [24,28,31,37]). Since the human vascular response to NO<sub>3</sub><sup>-</sup> supplementation is dependent on efficient bacterial reduction of NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup>, it is possible that at least some of this variability is linked to differences between individuals in the oral microbiota and therefore oral NO<sub>3</sub><sup>-</sup> reducing capacity. NMDS analysis revealed no overall differences in the salivary microbiomes between young and old participants, or following placebo or NO<sub>3</sub><sup>-</sup> supplementation. The greater responsiveness to supplementation in the old compared to young participants was surprising, given that ageing is typically associated with reduced salivary flow rate and altered oral bacterial colonisation [33]. It is important to note that although the self-reported frequency of dry mouth symptoms was greater in the young adults, we did not directly quantify salivary flow rate in this study, and agreement between self-reported xerostomia questionnaire

data and measured saliva secretion rate varies [11,29,30]. We are therefore unable to determine whether the higher occurrence of self-reported symptoms of dry mouth in the young adults was in fact attributed to significantly lower salivary flow in comparison to the old adults. Whether possible differences in salivary flow rate and/or NO<sub>3</sub> uptake into the enterosalivary circulation via sialin 2NO<sub>3</sub><sup>-</sup>/H<sup>+</sup> transporters [34] contribute to inter-individual variability in responsiveness to NO<sub>3</sub><sup>-</sup> supplementation irrespective of age warrants further investigation with large cross-sectional cohorts across the human lifespan. Physical exercise and diet are emerging as powerful modulators of the gut microbiota [1] and it is intuitive that the microbiota in the oral cavity, the uppermost section of the alimentary canal, may also vary according to age, diet, and with physical activity levels. There is evidence to suggest that the gut microbiome of healthy older people may be remarkably similar to that of young adults [4], and that age-related alterations in the gut microbiome may be related to advancing frailty and development of disease [3]. Whether the maintenance of a 'young' gut microbiome into older age is a cause or consequence of healthy ageing is unknown. Our data suggest that the oral microbiome of individuals who have reached their 8th decade of life without chronic disease is indistinguishable from oral microbiome of young adults. The inclusion criteria in the present study meant that the enrolled old participants were in exceptionally good health for their age and

therefore not representative of an ageing population with poor cardiovascular and/or metabolic health, who may be less responsive to NO<sub>3</sub> supplementation due to impairments in NO bioactivity [35]. Indeed, we found that there was no difference in blood pressure between young and old participants following NO<sub>3</sub><sup>-</sup> supplementation. Longitudinal studies are necessary to identify possible changes that may occur in the oral microbiome, and NO bioavailability, at the onset of chronic disease. The dietary interventions in the present study consisted of twice daily ingestion of NO<sub>3</sub><sup>-</sup>-rich and NO<sub>3</sub><sup>-</sup>-depleted beetroot juice concentrate that were matched for carbohydrate and polyphenol content [12]. It should be noted that the alterations we observed in the salivary microbiome following NO<sub>3</sub><sup>-</sup>-rich beetroot juice supplementation may be due to NO<sub>3</sub><sup>-</sup> alone or an additive effect of NO<sub>3</sub><sup>-</sup> and other nutritional components of the beetroot supplement. Elevated carbohydrate intake may have favoured the growth of those microbes that use carbohydrate as an energy substrate at the expense of proteolytic bacteria, such as *Prevotella*. Further studies using supplementation with salts of NO<sub>3</sub><sup>-</sup> (KNO<sub>3</sub>, NaNO<sub>3</sub>) are warranted to ascertain the effect of NO<sub>3</sub><sup>-</sup> alone on the oral microbiome.

#### 4.4. Methodological considerations

Microbial analysis was performed on tongue swab samples at baseline and on saliva samples following placebo and NO<sub>3</sub><sup>-</sup> supplementation periods. Saliva samples represent a composite of bacteria from all oral sites, while tongue swab samples target the tongue dorsum which was shown to have the highest NO<sub>3</sub><sup>-</sup> reductase activity by Doel et al. [10]. The quantities and relative abundances of bacteria vary between different oral sites, such that potential changes in microbiome assessed from tongue swabs following NO<sub>3</sub><sup>-</sup> supplementation may differ from those observed in the saliva samples in the present study. However, given the similarities of findings on *Neisseria* and *Rothia* between the present study and that of Velmurugan et al. [39], we are confident that the salivary microbiome analyses in the present study captured representative and meaningful differences between conditions in the NO<sub>3</sub><sup>-</sup> reducing oral microbiome. A limitation of the current study was that

saliva samples could not be included at the beginning of each supplementation period, and the wash-out period required for NO<sub>3</sub>-induced changes in the oral microbiome to return to baseline following cessation of supplementation is not known. Future work should include in vivo tests of oral NO<sub>3</sub>- reduction capacity to ascertain whether the changes in oral microbiome following chronic NO<sub>3</sub>- supplementation are associated with enhanced oral NO<sub>3</sub>- reduction. In the present study, we inferred enhanced oral NO<sub>3</sub>- reduction from plasma [NO<sub>2</sub>-]. Finally, further investigation should target the optimisation of the dose and duration of prebiotic NO<sub>3</sub>- supplementation, and its possible interactions with the macronutrient content of the diet, to provide maximal functional effects of NO<sub>3</sub>- supplementation.

#### 4.5. Conclusions

Imbalances in the oral microbial community and poor dental health have been associated with reduced cardiovascular and metabolic health. We showed that ageing, per se, in the absence of chronic disease, does not impair an individual's ability to reduce dietary NO<sub>3</sub>- and increase plasma [NO<sub>2</sub>-] in response to NO<sub>3</sub>- supplementation. Using 16S rRNA gene sequencing of oral bacteria in an in vivo experimental model, we showed that high abundances of oral bacteria belonging to genera *Prevotella* and *Veillonella* were likely detrimental, while high abundances of the genera *Rothia* and *Neisseria* were likely beneficial for the maintenance of NO homeostasis and associated indices of cardiovascular health. The symbiotic relationship between the oral microbiome and its human host is a fast evolving field of research with significant implications for development of prebiotic and probiotic interventions to improve cardiovascular and metabolic health. Our results identify

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dietary NO<sub>3</sub>- as a modulator of the oral NO<sub>2</sub>-producing microbiome in healthy humans and highlight the potential of oral microbiota-targeted therapies for ameliorating conditions related to low NO bioavailability.

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Frequency of Tongue Cleaning Impacts the Human Tongue Microbiome Composition and Enterosalivary Circulation of Nitrate



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The oral microbiome has the potential to provide an important symbiotic function in human blood pressure physiology by contributing to the generation of nitric oxide (NO), an essential cardiovascular signaling molecule. NO is produced by the human body via conversion of arginine to NO by endogenous nitric oxide synthase (eNOS) but eNOS activity varies by subject. Oral microbial communities are proposed to supplement host NO production by reducing dietary nitrate to nitrite via bacterial nitrate reductases. Unreduced dietary nitrate is delivered to the oral cavity in saliva, a physiological process termed the enterosalivary circulation of nitrate. Previous studies demonstrated that disruption of enterosalivary circulation via use of oral antiseptics resulted in increases in systolic blood pressure. These previous studies did not include detailed information on the oral health of enrolled subjects. Using 16S rRNA gene sequencing and analysis, we determined whether introduction of chlorhexidine antiseptic mouthwash for 1 week was associated with changes in tongue bacterial communities and resting systolic blood pressure in healthy normotensive individuals with documented oral hygiene behaviors and free of oral disease. Tongue cleaning frequency was a predictor of chlorhexidine-induced changes in systolic blood pressure and tongue microbiome composition. Twice-daily chlorhexidine usage was associated with a significant increase in systolic blood pressure after 1 week of use and recovery from use resulted in an enrichment in nitrate-reducing bacteria on the tongue. Individuals with relatively high levels of bacterial nitrite reductases had lower resting systolic blood pressure. These results further support the concept of a symbiotic oral microbiome contributing to human health via the enterosalivary nitrate-nitrite-NO pathway. These data suggest that management of the tongue microbiome by regular cleaning together with adequate dietary intake of nitrate provide an opportunity for the improvement of resting systolic blood pressure.

#### Introduction

The human oral cavity is an important habitat for microbes, and a healthy mouth can harbor upwards of ten billion bacteria (Loesche, 1993). Cardiovascular research has identified a potential role for the oral microbiome in human health via the conversion of dietary nitrate into nitrite, the bioactive storage pool available for spontaneous conversion to nitric oxide (NO) (Lundberg and Govoni, 2004; Lundberg et al., 2008). Continuous generation of NO is essential for the integrity of the cardiovascular system, and decreased production or bioavailability of NO

is central to the development of many heart-related disorders (Lundberg et al., 2004, 2009; Bryan and Loscalzo, 2017). The human body is able to produce NO directly by the five-electron oxidation of L-arginine (Moncada and Higgs, 1993) by endothelial nitric oxide synthases (eNOS; Figure 1A). However, the eNOS gene is polymorphic, and the pathway can become dysfunctional with age (Niu and Qi, 2011). Failure to produce sufficient NO is causal for the onset and progression of a number of cardiovascular diseases, including hypertension and atherosclerosis (Taddei et al., 2001; Torregrossa et al., 2011). The enterosalivary nitrate-nitrite-NO pathway in humans appears to serve as an alternative pathway for production of bioactive NO, supplementing host endothelial NO production. This pathway functions via the bacterial conversion of dietary nitrate into nitrites that can then be converted to NO and participate in the regulation of endothelial vasodilation (Figure 1B) (Benjamin et al., 1994; Lundberg et al., 1994). This diet-dependent pathway relies upon commensal oral bacteria located on the tongue dorsum to perform the first step (nitrate reduction to nitrite) since mammals lack a functional nitrate reductase (Lundberg et al., 2004; Doel et al., 2005).

FIGURE 1

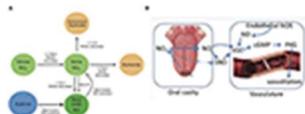


Figure 1. Enterosalivary circulation of nitrate. (A) Bacterial and mammalian enzymes in nitrate metabolism. The bio-activation of nitrate from dietary or endogenous sources requires its initial reduction to nitrite, and because mammals lack nitrate reductase enzymes, this conversion is mainly carried out by commensal bacteria. The presence of bacteria encoding nitrate reductase (1.7.99.4) is predicted to be critical for enterosalivary circulation. Nitrite represents a major storage form of NO in blood and tissues: Nitrite-NO conversions can be driven by enzymatic and chemical mechanisms. 1.7.99.4—Nitrate reductase, 1.7.2.1—Nitrite reductase (NO-forming), 1.7.2.2—Nitrite reductase (cytochrome; ammonia-forming), 1.7.1.4—Nitrite reductase (NAD(P)H). (B) Interactions of oral nitrate metabolites with the host vasculature. Dietary nitrate converted to nitrite in the oral cavity may be converted to NO and/or react with endothelial and plasma proteins to form S-nitrosothiol (SNO). Nitrite along with NO and SNO has been shown to activate soluble guanylyl cyclase (sGC) and increase cGMP levels in tissue. Nitrite-NO-SNO, whether from dietary sources or endothelial production, improves vascular tone via cell signaling through cGMP/PKG and stimulation of smooth muscle relaxation. Although there is compelling evidence supporting the role of the tongue microbiome in the enterosalivary nitrate-nitrite-NO pathway, this remains a relatively unexplored area of human-microbial mutualism and many important questions must be addressed. Previous studies have demonstrated that nitrite-induced reductions in blood pressure are inhibited by antiseptic chlorhexidine (CHX) or other antibacterial mouthwashes (Tannenbaum et al., 1976; Govoni et al., 2008; Petersson et al., 2009; Kapil et al., 2013; McDonagh et al., 2015; Woessner et al., 2016; Mitsui and Harasawa, 2017). The focus of these studies has been from the perspective of cardiovascular physiology, and little information is available regarding the oral health status or habits of subjects in these studies. In this study we approach this symbiotic relationship from the perspective of oral health using a cohort of 27 oral health professionals with both excellent cardiovascular and oral health. These subjects used a CHX mouthwash for 7 days, and we assessed the response of resting systolic blood pressure and tongue microbiome community

composition before and after exposure to this potent antimicrobial agent. The human oral microbiome is highly variable between individuals; therefore, we hypothesized that there would be inter-subject variability in response to CHX dependent upon the composition of the baseline tongue microbiota. In this study we provide the first assessment of the tongue microbiome in parallel with resting blood pressure in healthy individuals treated with CHX.

## Materials and Methods

### Subject Recruitment

Subjects were recruited from the faculty, staff, dental and dental hygiene students of the University of Texas Health Science Center at Houston School of Dentistry. For inclusion in the study, subjects were over the age of 18 and capable of giving consent, were non-smokers, had not used antibiotics within the previous 3 months, had no history of bone loss, and no history of hypertension. Volunteers were evaluated for oral health, including the use of a standard periodontal exam, with spot probing for bleeding and loss of attachment, and an oral health subject history. During assessment, subjects were excluded upon discovery of bleeding on probing at more than 10% of sites, <24 teeth, attachment loss of more than 4 mm at any site, the presence of oral hard or soft tissue lesions, or a resting blood pressure of >130 mm/Hg. Thirty four subjects were screened, and 6 subjects excluded after oral exam and initial blood pressure measurements (Supplemental Data Figure 1). Of the 28 subjects initiating the study, one was discontinued due to antibiotic usage at the first follow-up visit, and one (subject NiOx38) was discontinued at the final visit. Data from the first three time points for NiOx38 were used for data analysis. Twenty-six subjects completed the final visit of the study. The study protocol was approved by the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston (HSC-DB-14-0078). Subject demographic data is shown in Table 1.

TABLE 1

	Count	Percent
<b>SEX</b>		
Male	10	37%
Female	17	63%
<b>AGE</b>		
20-30	10	38%
30-40	6	22%
40-50	3	11%
50 and over	2	8%
<b>RACE AND ETHNICITY</b>		
African American	3	10%
Caucasian	11	40%
Asian	5	17%
Hispanic	6	21%
Middle Eastern	1	3%
Asian-Caucasian	1	3%
<b>EDUCATION</b>		
High School Diploma	1	4%
Some college	5	19%
Associate Degree	1	4%
Bachelor's Degree	11	41%
Professional Degree	8	30%
No response	1	4%

27 subjects were enrolled in the study, and distribution of subjects by major demographic measures are shown.

Table 1. Demographics.

### Study Design And Procedures

The study uses a repeated measures linear mixed model to detect changes in systolic blood pressure over 14 days. Power analysis estimated that 24 subjects needed to complete the study in order to detect a medium effect size at 80% power. The study uses longitudinal repeated measures of resting systolic blood pressure as the primary outcome. The study protocol consisted of 4 visits: day 1 (baseline), day 7 (after 7 days CHX treatment), day 10 (3 days recovery) and day 14 (7 days recovery). Study visits were always scheduled at noon, and lasted for 1 h to allow repeated resting blood pressure measurements. For the duration of each visit,

subjects were seated in a dental chair in the upright position, with ankles uncrossed. At the first visit, the subjects completed general demographics and medication questionnaires, and an oral hygiene survey.

At each visit blood pressure measurements were taken three times with an interval of 15 min between the measurements. The first blood pressure reading was taken after at least 10 min seated in the chair (Kallioinen et al., 2017). Blood pressure was taken on alternating arms at each time point, beginning with the right arm. Tongue scrapings and saliva were collected at each visit. Unstimulated whole saliva was taken using the passive drool method into a cryovial with a saliva collection aid (Salimetrics, LLC). Tongue samples were collected by drawing a sterile stainless steel tongue scraper with gentle pressure across the tongue dorsum from the posterior to the mid-anterior. 50 ul of tongue sample was transferred to MoBio PowerSoil tubes for bacterial DNA extraction and 16s rRNA community analysis. The remainder of the sample was transferred to cryovials containing freezing medium [(0.045% K<sub>2</sub>HPO<sub>4</sub>, 0.045% KH<sub>2</sub>PO<sub>4</sub>), 0.09% NaCl, 0.09% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.018% MgSO<sub>4</sub>, 0.038% EDTA, 0.04% Na<sub>2</sub>CO<sub>3</sub>, 0.02% dithiothreitol, 0.2% Bacto-agar, 5% glycerol] and stored at -80°C for preservation of viable bacteria. At each visit, after collection of oral samples, subjects performed a nitrate rinse for 2 min with 50 ml of a solution of 1 mM sodium nitrate to assess in vivo nitrate reduction by oral bacteria. After 2 min, subjects spit the 50 ml nitrate rinse sample into a collection tube. Samples were frozen at -80°C, and subsequently analyzed for nitrate/nitrite content as described in Nitrate-Nitrite Quantification in Subject Samples.

The treatment in this study was use of 0.12% Chlorhexidine gluconate mouthwash (Peridex) twice a day for 7 days. At the completion of the first visit each subject was provided with a prescription oral mouthwash with 0.12% Chlorhexidine gluconate (CHX) and instructed to rinse with ½ ounce for 30 s twice a day as part of their normal oral hygiene in the morning and evening. Subjects were asked to discontinue use of any other mouthwash for the first 7 days, but otherwise maintain their normal oral care regimen, as documented by the oral hygiene survey (Table 2). At their second visit (day 7 time point) subjects were instructed to cease use of CHX and return to their normal oral health care products. Subjects returned at day 10 and 14 for blood pressure and oral sampling during the recovery from treatment phase.

TABLE 2

	Count	Percent
<b>BRUSH TEETH</b>		
Twice a day	22	81%
Three times a day	5	19%
<b>FLOSS</b>		
Several times per month	1	4%
Several times per week	8	30%
Once a day	16	61%
Twice a day	2	7%
<b>CLEAN TONGUE</b>		
Less than once a week	3	12%
Weekly	1	3%
Once a day	13	48%
Twice a day or more	10	37%
<b>MOUTHWASH USE</b>		
Always/often	5	19%
Once a day	16	59%
Twice a day	6	22%
<b>MOUTHWASH INGREDIENT</b>		
Essential oils	11	41%
Cetylpyridinium chloride	10	37%
No response	6	22%
<b>TYPE OF TOOTHBRUSH</b>		
Manual	10	37%
Electric	12	44%
Both manual and electric	5	19%
<b>VISITS TO DENTIST PER YEAR</b>		
None	1	4%
Once	12	44%
Twice or more	14	52%

Subject responses to the oral hygiene survey are summarized.

Table 2. Oral hygiene survey.

Bacterial Viable Counts and Nitrate Reduction Assay

A subset of six subjects performed an 8-h time course to assess bacterial response to CHX. Tongue samples were obtained from the dorsum using a stainless steel tongue scraper hourly for 8 h after rinsing with CHX. A total of 10 samples were collected, at baseline, after CHX rinse, and hourly afterward for 8 h. Bacterial samples were collected from the posterior of the tongue dorsum by gently stroking the surface with a 0.6 cm diameter metal scraper three times, over a ~0.6 cm<sup>2</sup> surface area.

A different section of the tongue was sampled at each time point. Bacterial sample was transferred to a cryovial containing freezing medium and stored immediately at -80°C for preservation of viable bacteria. For calculation of viable counts, samples were thawed on ice and serially diluted five times at 20-fold increments. The final three dilutions were dropped in 5 ul aliquots in triplicate onto Trypticase Soy Blood agar supplemented with 0.1%Yeast/7.5 uM hemin/3 uM vitamin K (TSBY-S) and incubated anaerobically at 37°C for 72 h. For calculation of ex-vivo nitrate reducing activity in tongue samples, samples were standardized to the same starting OD660 in sterile saline, and 100 ul aliquots were grown in 24 well tissue culture plates with 1 ml of TSBY-S broth supplemented with 5 mM nitrate solution. The plates were incubated for 24 h anaerobically and statically at 37°C. Biofilm supernatants were collected and processed for nitrite-nitrate measurements using the Nitrate reduction test (Sigma-Aldrich) as recommended.

#### Blood Pressure Statistical Analysis

Blood pressure data was analyzed using repeated measures two-way ANOVA, for both systolic and diastolic readings. The first factor was time point, and the second factor was subject. Additional analysis was done with the second factor as gender, race-ethnicity, and other demographic variables. Post-hoc tests between time points and within individuals were done with Bonferroni analysis and Fishers Least Significant Difference. Association of blood pressure changes with subject metadata was assessed by Spearman's correlation. Differences between population distributions were assessed by the F-test for Variances. Power analysis for study design was done with G\*Power. All other statistical analysis and graphing were performed with StatPlus for Excel (Microsoft).

#### Nitrate-Nitrite Quantification in Subject Samples

Nitrate and nitrite were determined by using HPLC, as described previously (Rassaf et al., 2002; Bryan and Grisham, 2007; Jiang et al., 2012b). This method employs ion chromatography with on-line reduction of nitrate to nitrite and subsequent post-column derivatization with the Griess reagent (ENO-20, EiCom, Kyoto, Japan).

#### Bacterial Community Analysis

##### DNA Extraction and 16S rRNA Gene V4 Amplification and Sequencing

Bacterial DNA was extracted from tongue samples using MoBio PowerSoil (Qiagen) protocol with 0.7 mm garnet beads, as recommended by the manufacturer. The V3-V4 region of the bacterial genomic DNA was amplified using barcoded primers 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3'). The barcoding PCR reaction contained the following: 2 uL 4 uM barcoded primer stock, 5 uL DNA, 2 uL Taq Buffer II (Invitrogen), 0.15 uL Taq enzyme (Invitrogen), and 10.85 uL PCR. The reactions are amplified in an Eppendorf mastercycler Thermocycler under the following conditions: initial denaturation step for 2 min at 95°C, followed by 30 cycles of 20 s denaturation at 95°C, 45 s of annealing at 50°C, and 90 s annealing at 72°C. A different barcode is used for each sample, allowing for

pooling of samples for sequencing. All samples were pooled and sequenced on one lane of an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA) at the Baylor College of Medicine Human Genome Sequencing Center.

#### 16S rRNA Gene Data Analysis

Reads 1 and 2 from the Illumina-sequenced amplicons were de-multiplexed and imported as paired reads into CLC Genomics Workbench v10 (Qiagen), trimming adaptors and barcodes. Reads were merged using alignment parameters with a mismatch score of 2, a minimum total score of 8, and no tolerance of unaligned ends, and reads that failed to merge were discarded. Final reads were between 250 and 480 bp in length. Comparative quality of samples was assessed with the “filter samples” function, requiring all samples in the study to fall within 50% of the median number of reads to pass. All subject samples in the study passed the filter, with total reads per sample between 28,000 and 58,000. Sequences were clustered into OTUs against the HOMD 16s rRNA RefSeq database at 98% identity (version 14.5) (Chen et al., 2010). OTU clustering was set to allow creation of novel OTUs that failed to meet the similarity percentage required to match the selected database. The minimal occurrence to be defined as an OTU was set to 2. Novel OTUs were assigned identity by BLAST comparison to the bacterial 16s rRNA database at NCBI. Chimera detection was performed as part of OTU clustering with a chimera crossover cost of 3 using a Kmer size of 6. OTUs identified as chimeras were removed from the final OTU abundance table. Metadata was added to the final OTU abundance table to allow aggregation of data by time point, gender, subject, race, or oral hygiene frequency. Bar or pie charts were constructed to visualize the taxa present in each sample and across sample groups.

#### Calculation of Alpha and Beta Community Diversity and Significant Differences

Community diversity was assessed using the Microbial Genomics Diversity module of CLC Genomics Workbench. OTUs from the abundance table were aligned using MUSCLE with a required minimum abundance of 10. Aligned OTUs were used to construct a phylogenetic tree using Maximum Likelihood Phylogeny using the Neighbor Joining method and the Jukes Cantor substitution model. Alpha diversity measures were calculated for total OTUs, and Shannon's Entropy. Rarefaction analysis was done by sub-sampling the OTU abundances in the different samples at a range of depths from 1 to 100,000; the number of different depths sampled was 20, with 100 replicates at each depth. Statistical significance in alpha diversity between cohorts and time points was calculated with one-way ANOVA and post-hoc tests by Bonferroni.

PERMANOVA Analysis (Permutational Multivariate Analysis Of Variance) was used to detect significant differences in Beta diversity between groups. Differential abundance tests (non-parametric ANOVA) on the OTU frequency table were used to identify significant changes in the relative abundances of individual OTUs between groups. Differential abundance analysis values were calculated for: the max group means (maximum of the average RPKM's),  $-\log_2$  fold change, fold change, standard p-value (significance at  $<0.05$ ), and FDR p-value (false discovery rate corrected p-value).

#### Community Gene Content Using PICRUSt And LEFSE

Sequences were clustered into OTUs against the Greengenes database at 97% homology, and the minimal occurrence to be defined as an OTU was set to 2. OTU's that failed to match the database were discarded. Data tables were clustered by tongue cleaning frequency and time point, or dominant genus and time point, and relative abundance tables were imported into

PICRUSt for estimation of community gene content

(<http://huttenhower.sph.harvard.edu/galaxy>) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999). Significant enrichment of enzyme functions was identified using LEFSE (Segata et al., 2011).

Quantitative RT-PCR for Copper-Containing Nitrite Reductase (EC 1.7.2.1)

DNA sequences for the nitric oxide-forming nitrite reductase gene [KEGG orthology K00368; (Cantera and Stein, 2007)] were downloaded from 18 strains of *H. parainfluenza*, 1 strain of *H. pittmaniae*, 2 strains of *H. parahaemolyticus*, and 9 commensal strains of *Neisseria* (*N. mucosa/sicca/subflava/weaveri/cinerea*). The genes were aligned with the “create alignment” module of CLC Genomics, with a gap open cost of 10 and a gap extension cost of 1.0. The resulting alignment is shown in Supplemental Data Figure 8A, and using the “design primers” module, multiple pairs of universal degenerate primers were designed. These universal primers failed specificity testing by BLAST against whole bacterial genomes. A second round of genus specific universal primers were designed for the *Haemophilus* nitrite reductase gene *nirK*, and the *Neisseria* nitrite reductase gene *aniA*. These primers passed both BLAST specificity and PCR cross-testing against genomic DNA of *Haemophilus* oral taxon 851 strain # F0397 and *N. mucosa* strain C102 (BEI Resources, Manassas, VA) by melting curve analysis and agarose gel analysis. Other quality control testing for was performed as recommended for qRT-PCR by the manufacturer (Taylor et al., 2010). Universal PCR primers for bacterial 16s rDNA were 907R and 357F as previously described (Martin et al., 2002). All primers were synthesized by Sigma-Aldrich, The Woodlands, TX. Quantitative RT-PCR reactions were carried out using SsoAdvanced Universal SYBR Supermix (Bio-rad, Hercules, CA). Reactions were performed on a StepOnePlus Real-Time PCR System (ThermoFisher Scientific), in 20  $\mu$ l reactions with 10  $\mu$ l 2 $\times$  Supermix, 1–5  $\mu$ l template, and 300 nM of each primer. Standards were 10-fold serial dilutions of genomic DNA from *Haemophilus* oral taxon 851 strain # F0397 (for *Haemophilus* nitrite gene *nirK* PCR), or genomic DNA from *N. mucosa* (for *Neisseria aniA* nitrite gene and 16s rRNA PCR). Genome equivalents per ng of genomic DNA were calculated on a genome size of 2.3 mB for *Neisseria* and 2.0 mB for *Haemophilus*. Standards were run with triple technical replicates, unknowns and negative controls were run in duplicate. Each primer set was run on separate 96 well plates, and each primer set was run at least twice. The relative ratios of nitrite gene counts normalized to bacterial cell number were calculated as (*Haemophilus* gene counts + *Neisseria* gene counts) divided by total 16s gene counts.

## Results

### Blood Pressure Changes in Response to Treatment With and Recovery From Chlorhexidine

The subjects recruited for this study were all faculty, staff, or students at the University of Texas School of Dentistry at Houston. The study design consisted of a baseline visit, a visit after 7 days treatment with CHX, a 3-days recovery from treatment follow-up, and a 7-days follow-up (Supplemental Data Figure 1). Twenty-seven subjects completed the first stage of the study, and 26 subjects completed the entire study. There were 17 females and 10 males; the average age was 31.8 years, with the youngest subject 22 and the oldest 71 years of age (Table 1). The average resting blood pressure at baseline in this cohort was 113/78 mmHg, and both systolic and diastolic data points conformed to a normal distribution. Two-way repeated measures ANOVA of systolic blood pressure readings indicate that significant differences exist between time points ( $p = 0.017$ ), and between individual subjects ( $p = 0.0001$ ), with a significant

interaction component ( $p = 0.005$ ). Post-hoc analysis identified significant changes in systolic blood pressure between the treatment and 3-days recovery time points (115 vs. 111.5 mm Hg), as well as the 3-days recovery and 7-days recovery (111.5 vs. 113.3 mm Hg; Table 3). Diastolic blood pressure was also significantly different at these same points.

TABLE 3

Variable	Baseline	Treatment	3 Days Recovery	7 Days Recovery
Systolic Blood Pressure (mm Hg)	115.0	111.5	113.3	113.3
Diastolic Blood Pressure (mm Hg)	75.0	71.5	73.3	73.3
Mean Systolic Blood Pressure (mm Hg)	115.0	111.5	113.3	113.3
Mean Diastolic Blood Pressure (mm Hg)	75.0	71.5	73.3	73.3

Table 3. Blood pressure values.

#### Individual Blood Pressure Responses to Treatment With Chlorhexidine

The time point-subject interaction  $p$ -levels indicate that there were significant individual differences in response to CHX. Assessment of systolic blood pressure results on within-subject data revealed that 13 subjects had changes in blood pressure  $>5$  mm/Hg in response to CHX; 9 subjects had an increase in resting blood pressure after treatment with CHX, while four had a decrease (Figure 2). This bimodal response to CHX treatment was confirmed by comparison of the blood pressure distribution at baseline to the other time points by an F-test for Variances. Only the CHX treatment time point has significantly different variance compared to baseline ( $p$ -level two-tailed = 0.04). Thus, in our study, there is a significant difference in individual response after 7 days of treatment with CHX, and an overall significant decrease in systolic and diastolic blood pressure average in the cohort after recovery from CHX for 3 days. We next determined if any of our demographic or hygiene data could account for the difference in individual response after 7 days of treatment with CHX.

FIGURE 2

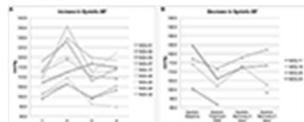


Figure 2. Individual systolic blood pressure responses. Individuals with a  $>5$  mm/Hg change in blood pressure after CHX treatment are shown (A) Nine subjects had an increase in systolic BP. (B) Four subjects had a decrease in systolic BP. Each point represents an average of three systolic readings of resting BP. Subject NiOX 11 did not have BP readings taken at the third time point.

#### Correlation Between Blood Pressure Response to CHX and Oral Hygiene Behaviors

At the first visit, subjects completed a demographic and oral hygiene survey. Subjects provided information on their age, gender, education level, number of times per day they brush their teeth, use dental floss, use mouthwash, clean their tongue, visit the dentist per year, and the types of oral care products used (Table 2). We utilized this data to investigate the different responses to treatment with CHX within our subject population. Both systolic and diastolic blood pressure was found to vary significantly by gender, with male subjects averaging 10 mm/Hg higher systolic and 5 mm/Hg higher diastolic than females. However, there was no significant interaction between gender and blood pressure response over the course of the study, indicating that there is no difference in response between males and females

(Supplemental Data Figure 2). No significant variation was found between any other groups based on race/ethnicity, education, or other variables.

Subject data, represented by change in systolic blood pressure between baseline and after the 1 week CHX treatment, was correlated with metadata categories using Spearman's correlation. No significant correlations were found for any demographic or oral hygiene data, except for tongue cleaning frequency. An R of 0.45 for correlation with tongue cleaning frequency was determined to be significant with a p-value of 0.03 (Figure 3A). Subjects who cleaned their tongue twice or more per day as part of their normal oral hygiene were more likely to have an increase in systolic blood pressure during use of CHX for 1 week. Subjects who did not clean their tongue on a daily basis were more likely to have a decrease in systolic blood pressure. Separating the subjects into cohorts based on tongue-cleaning frequency, we performed one-way ANOVA on blood pressure data by cohort, and confirmed the cohort of subjects who clean twice a day had a significant increase in systolic BP after 7 days CHX treatment, followed by a significant decrease at the 3-days recovery time point (Supplemental Data Figure 3, p-value = 0.003). The zero cleaning cohort shows the opposite response, with a significant decrease after 7 days CHX (117.5 mm/Hg to 111.0 mm/Hg, p-value = 0.03), and a subsequent increase at the 3 days recovery (111.0–117.4). To confirm that the frequency of tongue cleaning was not tied to other demographic variables, we used the Chi Square test for gender, age-range and race-ethnicity, with no significant associations.

FIGURE 3

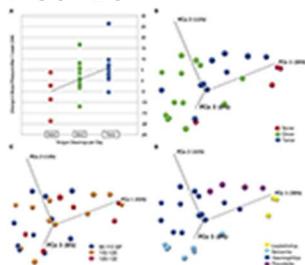


Figure 3. Tongue cleaning impact on blood pressure response and microbiome composition. (A) Correlation plot for blood pressure response to chlorhexidine and frequency of tongue hygiene. Spearman's correlation coefficient was used to determine the strength of association. (B) Bray-Curtis Principle component plot of bacterial communities at baseline, colored by tongue-cleaning frequency. (C) Bray-Curtis Principle component plot of bacterial communities at baseline, colored by resting systolic blood pressure. (D) Bray-Curtis Principle component plot of bacterial communities at baseline, colored by dominant bacterial genus.

From these observations, we hypothesized that the frequency of tongue cleaning as a significant effector of blood pressure could result from a combination of possible effects. First, regular tongue cleaning may result in a baseline tongue microbiome that has a greater ability to reduce nitrate, and conversely, failure to clean the tongue daily may result in a microbiome composition that is unfavorable to nitrate reduction. Further, cleaning the tongue disrupts the papillary surface and could allow increased penetration of CHX, resulting in a greater community disruption in the two cleaning cohort. To investigate these mechanisms, we used 16S rDNA community analysis of tongue samples to elucidate the dynamics of the tongue microbiome in our subjects, to compare differences between time points and tongue hygiene cohorts.

## The Bacterial Microbiome of the Healthy Human Tongue

In tongue samples from 27 subjects at baseline, 272 unique OTUs were identified at the phylum level, representing eight different phyla of bacteria: Proteobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, Candidate division SR1, Spirochaetes, and Candidate division TM7. Members of the first five phyla were found in every subject (Supplemental Data Figure 4A), while the remaining phyla were found as a minority component in some subjects, resulting in combined abundance values <1%. This is consistent with other studies in adults, which demonstrated the same major phyla on the tongue (Jiang et al., 2012a; Mark Welch et al., 2014). In our study, Proteobacteria account for 40% of the OTUs identified across the cohort; Bacteroidetes account for 23%, Firmicutes for 19% (Table 4). The most common OTU in our cohort at baseline was *Haemophilus parainfluenza*, a commensal bacterium of the oral-pharyngeal flora and a member of the Proteobacteria. This organism accounted for 22% of the combined abundance in the cohort, followed by *Neisseria subflava* at 12%. The genus *Haemophilus* was the most numerous in 14 of the 27 subjects at baseline (Supplemental Data Figure 4B). Six subjects had *Neisseria* as the most common genus, while four subjects had *Prevotella* and three had *Leptotrichia*. Other bacteria identified in this study are shown in Supplemental Data Figure 4C, and all species-level identifications are based on OTU comparisons to the HOMD 16s rRNA RefSeq database at 98% identity (Chen et al., 2010).

TABLE 4

Phylum/Genus	Average tongue microbiome composition by phylum	Average tongue microbiome composition by genus	High/low range
Proteobacteria	40%	25%	80-1%
<i>Haemophilus</i>			
Proteobacteria		13%	47-0.1%
<i>Neisseria</i>		2%	9-0%
Proteobacteria			
<i>Corynebacterium</i>	22%	18%	46-1%
Bacteroidetes			
<i>Prevotella</i>		3%	13-0%
Bacteroidetes			
<i>Haemophilus</i>		2%	5-0%
Firmicutes			
<i>Streptococcus</i>	19%	9%	20-2%
Firmicutes		6%	16-1%
<i>Staphylococcus</i>			
<i>Staphylococcus</i>		2%	16-0%
<i>Lactobacillus</i>		1%	2-0%
Firmicutes			
<i>Capsulobacterium</i>			
Fusobacteria	12%	6%	24-0%
<i>Leptotrichia</i>			
Fusobacteria		6%	19-0%
Fusobacteria			
<i>Actinobacterium</i>	5%	4%	13-0%
<i>Actinobacterium</i>			
<i>Actinobacterium</i>		1%	4-0%
<i>Mycobacterium</i>			

The first and second columns list the distribution of bacteria by phylum, in order of most common to least common. The third column shows the average composition by genus, and the last column indicates the highest percentage and lowest percentage for individuals in the study.

Table 4. Average composition of the tongue microbiome across all subjects.

### Effects of Tongue Hygiene and Demographics on Bacterial Community Composition

To test the effect of tongue cleaning behavior on microbiome composition, we generated Principle Component Plots for all samples at baseline. By PERMANOVA analysis, bacterial community distribution was significantly influenced by tongue cleaning frequency, with a Bray-Curtis ANOVA p-value of 0.00001, and Bonferroni post-hoc analysis indicated that all tongue-cleaning subgroups were significantly different from each other (Figure 3B). PERMANOVA values for systolic blood pressure ( $p = 0.009$ ) and dominant genus ( $p = 0.00001$ ) were also significant (Figures 3C,D). For systolic blood pressure, Bonferroni post-hoc analysis was significant only between groups 90–110 and 120–130 ( $p = 0.006$ ). To further assess the demographic and behavioral influences on community composition, we used Chi Square to assess their correlations with the dominant bacterial genus per subject (Supplemental Data

Figure 5). Frequency of tongue cleaning was found to have a significant correlation with the dominant bacterial genus on the tongue.

We next analyzed differences between hygiene cohorts at baseline with differential abundance analysis (Supplemental Data Table 1). The largest differences in composition were found between the zero cleanings cohort and the other two. There are significant differences in 33 different OTUs for subjects with zero cleanings/day compared to the other two groups, and only four OTUs different between the one and two-cleaning groups. Notably, the levels of *Leptotrichia* sp. are significantly higher in the zero-cleanings group, and the levels of two *Haemophilus* OTUs are significantly lower. Other OTUs lower in the zero-cleanings group are associated with *Veillonella parvula*, *Rothia muciliginosa*, and *Granulicatella adiacens*. Therefore, tongue-cleaning does have a significant impact on tongue bacterial community composition, and daily cleaning (either once or twice) results in an increased proportion of *H. parainfluenza* and other Proteobacteria, which are major nitrate and nitrite reducing species of the oral cavity (Hyde et al., 2014).

#### Bacterial Community Responses to CHX Treatment

We next tested the hypothesis that cleaning the tongue twice a day could influence blood pressure by causing a greater community disruption by CHX compared to the other cohorts. PERMANOVA analysis and Principle Component Plots for all time points demonstrated that samples continue to cluster by tongue-cleaning frequency (Figure 4A) after treatment with CHX. Further, there was no significant change in overall sample distribution for any tongue-cleaning cohort in response to CHX, based on PERMANOVA analysis (Supplemental Data Figure 6).

#### FIGURE 4

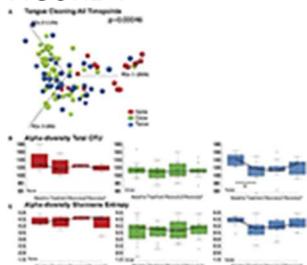


Figure 4. Effects of tongue hygiene and chlorhexidine exposure on community diversity and composition. (A) Bray-Curtis Principle component plot of bacterial communities across all time points, colored by tongue cleaning frequency. The distribution between cohorts remains distinct; the PERMANOVA  $p$ -value is 0.00046. (B) Community richness as measured by Total OTUs. Only the two-cleaning per day cohort, shown in blue, had a significant change in richness after CHX treatment as noted by the asterisk. (C) Community diversity as measured by Shannon entropy. No significant differences were noted for any cohort.

We further considered the impact of CHX exposure on community richness and diversity using Observed OTUs and Shannon Entropy calculations. Richness decreased upon CHX treatment for all three tongue-cleaning cohorts, but the only statistically significant decrease was in the two-cleaning group, between baseline and treatment with CHX (Figure 4B). Species diversity was also impacted in the two cleaning group after CHX exposure as assessed by Shannon Entropy, however this decrease was not statistically significant (Figure 4C).

Differential abundance analysis identified significant changes in some species in response to treatment with CHX (Supplemental Data Table 2). In the zero cleaning cohort three species had

significant changes in abundance, while the one cleaning cohort had 10 and the two cleaning had 12. Of the species that increase in abundance after treatment, six are from the genus *Capnocytophaga*, which are known to be more resistant to CHX than other oral microbes (Wade and Addy, 1989). Collectively, this data demonstrates that twice-daily tongue cleaning in combination with CHX treatment has an impact on tongue microbiome richness, and a larger magnitude of impact relative to the other tongue-cleaning cohorts. The primary effect of tongue cleaning in general, however, appears to be on selection of a baseline community of bacteria enriched in nitrate-reducing species.

#### Microbiome Viability and Metabolism After CHX Treatment

We were surprised to discover that CHX treatment did not cause large-scale changes in microbiome community structure, as it is a potent antimicrobial. We next assessed the effect of CHX exposure on tongue microbiome viability and nitrate reductase activity for 8 h after a 30 s rinse, on six subjects (Figure 5). CHX caused a significant reduction in bacterial viability, but this effect was only a 10-fold reduction, detectable 6 h after treatment. The dynamics of recovery from CHX exposure are notable, in that there was a rapid recovery in viable counts between 6 and 8 h, which corresponded with a significant increase in nitrate reduction to nitrite in the tongue samples. Thus, CHX mouthwash does not eradicate viable bacteria on the tongue, but instead causes a transient loss of viable counts, and the recovery from CHX treatment is associated with increased bacterial metabolic activity.

FIGURE 5

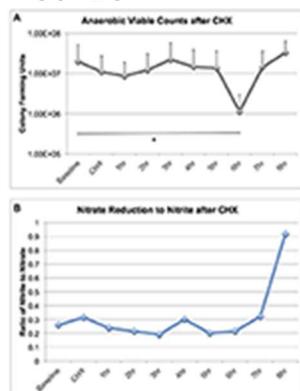


Figure 5. Bacterial viable counts and nitrate reductase activity recovered from tongue scrapings after CHX exposure. Six subjects collected tongue scraping samples over an 8 h time course after a 30 s rinse with 2% Peridex. Tongue samples were adjusted to a standardized starting OD600 and: (A) Serially diluted and plated for enumeration of viable counts on blood agar plates incubated anaerobically for 7 days and (B) Assessed for nitrate and nitrite values after anaerobic overnight incubation in 5 mM nitrate broth. Nitrate and nitrite results are shown as a ratio. Significant differences in viable counts between time points is indicated by an asterisk.

#### Blood Pressure and CHX Responses by Dominant Genus Cohorts

We next revisited our blood pressure data and community responses to CHX from the perspective of the bacterial community type, and noted that bacterial communities associated with the lowest systolic blood pressure (90–110 mm/Hg) were localized to the left side of the principle component plot, and superimposed more closely with the *Haemophilus* and *Neisseria* groups (Figures 6A,B). In support of this observation, we found that there were significant differences ( $p = 0.005$ ) between bacterial genus groups in systolic blood pressure at baseline

(Figure 6C). The three subjects with *Leptotrichia* tongue communities have an average systolic blood pressure at baseline of 123.2 mm/Hg, while the *Neisseria* defined-cohort has a systolic average of 110 mm/Hg. Post-hoc analysis identified the *Leptotrichia* cohort as having significantly higher systolic BP than *Haemophilus* and *Neisseria* groups. No groups had significant changes in BP over the time course, although the *Haemophilus* group had a five point increase in BP after CHX use.

FIGURE 6

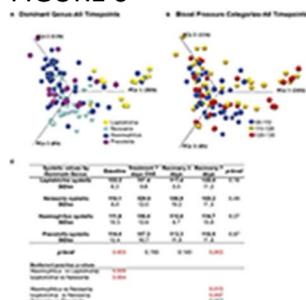


Figure 6. Effects of bacterial community and chlorhexidine exposure on systolic blood pressure. (A) Bray-Curtis Principle component plot of bacterial communities across all time points, colored by dominant bacterial genus. (B) Bray-Curtis Principle component plot of bacterial communities across all time points, colored by resting systolic blood pressure. (C) Statistical comparisons of systolic blood pressure between subjects, grouped by dominant bacterial genus.

#### Predicted Gene Content of the Human Tongue Microbiome

The PICRUSt program predicts gene family abundance (e.g., the metagenome) in microbial DNA samples for which only 16S rRNA gene data are available (Segata et al., 2011). Using PICRUSt, metagenomes were calculated for each tongue cleaning and bacterial genus cohort, and significant differences in relative abundance for gene families detected with linear discriminant analysis (LEfSe) with LDA score > 2 being considered significant. Across all samples, PICRUSt identified 4,295 genes with assigned KEGG orthology identifiers (KO). Comparing cohorts, 173 genes were found to be significantly different between tongue cleaning groups (Supplemental Data Table 3), and 674 genes between bacterial genus groups (Supplemental Data Table 4). Concerning the major bacterial enzymes associated with nitrate-nitrite metabolism (Figure 1A), NO-forming nitrite reductase [EC:1.7.2.1] varied significantly by both tongue-cleaning groups (Supplemental Data Figure 7B) and bacterial genus groups (Figure 7A); and [EC:1.7.2.2] ammonia-producing nitrite reductase was found to vary between the bacteria-genus cohorts (Figure 7B). Interestingly, the relative gene abundance for nitrate reductase did not significantly vary in either cohort (Supplemental Data Figure 7A). We confirmed the ability of all subjects to reduce nitrate to nitrite utilizing a 2-min in vivo oral rinse with 1 mM sodium nitrate. The average final concentration of nitrite for all subjects at baseline was 14  $\mu$ M, and as predicted by the gene abundance, there was no significant difference in nitrite generation from nitrate between tongue-cleaning groups (Supplemental Data Figure 7C;  $p = 0.3$ ) or bacterial-genus groups (data not shown).

FIGURE 7

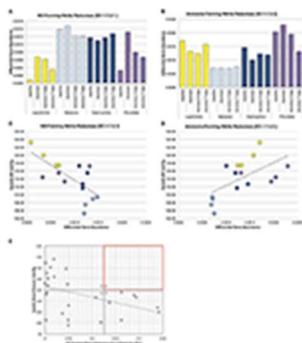


Figure 7. Relative gene abundance in the dominant genus cohorts. Subjects were grouped into cohorts based on microbiome type, named by the dominant genus found in the microbiome. (A) Predicted relative gene abundance for NO-forming nitrite reductase, which is significantly different between groups as determined by Lefse analysis. Predicted relative gene abundance was estimated by PICRUSt and represented as the percent of gene present in the community metagenome. (B) Predicted relative gene abundance for ammonia-forming nitrite reductase, which is significantly different between groups as determined by Lefse analysis. (C) NO-forming nitrite reductase gene abundance negatively correlated with changes in systolic blood pressure, with an  $R = -0.7$  and  $p = 0.002$ . (D) Ammonia-producing nitrite reductase positively correlated with systolic blood pressure, with an  $R = 0.6$  and  $p = 0.013$ . (E) The ratio of NO-forming nitrite reductase gene abundance in individual subjects at baseline, correlated with systolic blood pressure. The trend seen in the bacterial genus cohorts is reproduced in individual samples. The absence of subjects in quadrant 4 (red) implies that high ratios of NO-forming nitrite reductase on the oral microbiome may supplement host NO production and contribute to lower resting blood pressure.

We next performed correlation analysis across all cohorts at all time points for the NO-forming nitrite reductase gene. The gene did not correlate with blood pressure changes in the tongue cleaning cohort (Supplemental Data Figure 7D), but had a significant inverse correlation in the bacterial-genus cohorts (Figure 7C;  $R = -0.7$ ;  $p = 0.0025$ ). Additionally, the ammonia-producing nitrite reductase [EC:1.7.2.2] had a significant positive correlation with systolic BP (Figure 7D;  $R = 0.6$ ;  $p = 0.013$ ). Interestingly, although the *Neisseria* cohort and the *Haemophilus* cohort both have high levels of NO-forming nitrite reductase, the *Haemophilus* group has the higher amount of ammonia-producing reductase. Thus, high levels of NO-forming reductase are not sufficient, but rather high levels of NO-forming and low-levels of ammonia-forming produce the most favorable conditions for lower systolic BP.

#### Quantitative PCR Detection of NO-Forming Nitrite Reductase in Individual Subjects

The correlation between increased NO-forming nitrite reductase and reduced systolic BP in the bacterial genus cohorts was further investigated in individual subject samples from the baseline time point. Primers specific for the NO-forming nitrite reductase of *Haemophilus* and *Neisseria* were designed to derive the Proteobacteria contribution to NO-forming gene content from the tongue bacterial DNA (Supplemental Data Figures 8A–C). Further, the total bacterial content of each sample was determined using quantitative RT-PCR primers for bacterial 16s rRNA, and the ratio of the total NO-forming gene to 16s rRNA in the sample was calculated (Figure 7E). We observed a significant inverse correlation of  $-0.44$  ( $p$ -value = 0.02), further implicating a relationship between NO-forming bacterial nitrite reductase and resting systolic BP in individual

subjects. We further note that not all subjects require a high concentration of NO forming nitrite reductase to achieve a healthy blood pressure. Five subjects in quadrant 2 have a NO-forming reductase/16s ratio of  $<0.05$ , but have a resting systolic BP of  $<110$  mm/Hg. We predict that these subjects are producing sufficient endogenous NO, or have other BP regulation mechanisms in place that are bacterial NO-independent. In contrast, there are no subjects found in quadrant 4, implying that a high relative ratio of NO-forming nitrite reductase/16s ratio is contributing to a lower blood pressure.

#### Discussion

This is the first longitudinal next-generation sequencing study demonstrating the impact of oral hygiene on the composition of the tongue microbiome. Tongue microbiome communities are of general interest in Eastern medicine because the appearance of the tongue coating is considered a manifestation of systemic health (Jiang et al., 2012a). In Western medicine interest has focused on the role of the microbiome in mucosal disorders (Docktor et al., 2012; de Paiva et al., 2016), and in dentistry the tongue microbiome has significant associations with halitosis (Ren et al., 2016). Regular tongue cleaning is recommended by the American Dental Association (<http://www.mouthhealthy.org>) based on evidence that cleaning can reduce the severity of halitosis (Pedrazzi et al., 2016), however there are no epidemiological data on tongue cleaning practices or frequency in the United States population. Based on this study, tongue cleaning assumes a new importance from the perspective of blood pressure regulation, as daily tongue cleaning appears to favor the increased abundance and metabolic activity of nitrate/nitrite metabolizing species, such as *H. parainfluenza* and commensal *Neisseria* spp. (Barth et al., 2009).

Consistent with the theory that the enterosalivary nitrate-nitrite-NO pathway is foundational to cardiovascular health, we demonstrated that our cohort of healthy subjects uniformly have tongue microbiomes encoding nitrate reductase activity. Interestingly, introduction of the antiseptic mouthwash CHX revealed different blood pressure responses, and a significant component of this response was determined to be the frequency of tongue cleaning. From our analysis, regular tongue cleaning results in a tongue microbiome that has a greater ability to reduce nitrite to NO, and conversely, failure to clean the tongue daily results in a microbiome composition that is less favorable to NO production, but instead appears to favor conversion of nitrite to ammonia. Introducing CHX to patients who clean their tongue twice a day results in a significant disruption of community alpha-diversity compared to the other groups, linking bacterial community disruption to changes in blood pressure. Our study also demonstrates a chemostat-like activity of the tongue microbiome, in that loss of population due to CHX exposure stimulates a rapid population recovery (Rai et al., 2019). Rapid recovery parallels an increase in nitrate reduction metabolic activity, and we propose that frequent tongue cleaning may activate bacterial metabolism and benefit the host through production of nitrite. Thus, regular tongue hygiene both selects for a favorable microbiome and “revs up” the activity of the community.

Although we did not survey host diet as a variable in our study, the composition of the human diet clearly has an impact on the composition of the microbiome, both in the gut and in the oral cavity. In a recent study on the salivary microbiome comparing vegans and omnivores (Hansen et al., 2018), the authors determine that the ratio of *Neisseria* to *Prevotella* is significantly higher in vegans. Further emphasizing the importance of dietary nitrate on the oral

microbiome, Velmurugan et al. established in a randomized, double-blind study that sustained intake of nitrate-rich beetroot juice resulted in improved vascular function and also resulted in a significant increase in the percentage of *Neisseria flavescens* in the oral microbiome (Velmurugan et al., 2015). Both of these studies highlight the increase of commensal *Neisseria* spp. as a result of a diet high in nitrate-rich vegetables. Commensal *Neisseria* are part of the core oral microbiome in humans (Zaura et al., 2009), and reduction of nitrite to NO is obligatory for commensal *Neisseria* to survive in anaerobic conditions, such as those found in the crypts of the tongue (Bennett et al., 2014; Liu et al., 2015).

The data shown here provide static snapshots of what is undoubtedly a dynamic process. In our orally healthy cohort, the presence of nitrate reductase does not appear to be a rate-limiting component of the enterosalivary circulation of nitrate and subsequent reduction to nitrite. However, our experimental design has revealed a potential new aspect of enterosalivary circulation via the formation of NO in the oral cavity, in preference to formation of ammonia as the end product of nitrite. In subjects lacking other systemic health problems, the nitrite-reducing genetic content of the oral flora of the tongue significantly correlates with resting blood pressure values, with the putative mechanism through a supplemental contribution of nitrate reductase, and also nitric-oxide generating nitrite reductase. High levels of ammonia-forming nitrite reductase correlate with high blood pressure, and may result from “stealing” nitrite away from the NO pathway.

Although we have not established the mechanism by which generation of NO gas in the oral cavity could affect NO based vasodilation in the periphery, it is now well-established that NO generated in one biological compartment can affect NO homeostasis in distal tissue suggesting an endocrine function of NO (Elrod et al., 2008). Moreover, it is known that inhaled NO has systemic effects and can protect from myocardial ischemia reperfusion injury (Nagasaka et al., 2008) that is likely not due to NO itself but rather specific NO metabolites, nitrite and/or S-nitrosoglutathione (GSNO). Alternatively, NO gas produced in the lingual crypts could diffuse directly into the circulation through the highly vascularized tissue of the tongue.

Regulation of blood pressure is complex with multiple variables. There is a strong host component, including efficacy of endogenous NO production, weight, diet, gender, race, stress, and frequency of exercise (Rosendorff, 2013). We propose that the composition and metabolic activity of the oral microbiome should be considered an additional variable. Thirteen subjects on our study had changes of at least 5 mm/Hg in resting systolic blood pressure after CHX treatment, which is comparable to changes induced by manipulation of dietary salt intake (Graudal et al., 2017).

Manipulation of the human microbiome as a therapeutic target for disease management is on the near horizon. Screening the oral microbiome of resistant hypertensive patients may provide new insights into the etiology of their hypertension. The oral cavity is an attractive target for probiotic and/or prebiotic therapy because of the ease of access. The potential to restore the oral flora as a means to provide NO production is a completely new paradigm for NO biochemistry and physiology as well as to cardiovascular medicine and dentistry. These studies provide new insights into the host-oral microbiome symbiotic relationship. As NO is a ubiquitous signaling molecule, these systemic effects of these oral bacteria may have other significant effects on human health beyond maintenance of blood pressure.

Data Availability

16S rRNA gene data from this study is deposited at the Sequence Read Archive (SRA) maintained at <https://trace.ncbi.nlm.nih.gov/Traces/sra> under bioproject title "The Oral Microbiome in Blood Pressure Regulation" 2444628.

#### Ethics Statement

This study was carried out in accordance with the recommendations of the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston under study protocol number HSC-DB-14-0078. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

#### Author Contributions

GT, NA, and NB designed the study. NA, RW, B-YW, SE, IG, and KP recruited, screened, and sampled subjects. GT, D-HD, KR, NI, IS, and NB performed sample processing, nitrate-nitrite analysis, and quantitative RT-PCR. NJA and JP supervised bacterial DNA processing. GT performed bacterial community analysis. GT, NA, RW, B-YW, IS, EH, NJA, and NB wrote the manuscript.

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#### Conflict of Interest Statement

The authors of this manuscript declare the following competing interests: NB is a Founder and Shareholder of HumanN, a dietary supplement and functional nitric oxide nutrition company, a shareholder and consultant for SAJE Pharma, and he receives royalties on nitric oxide related patents from University of Texas. JP is President and Founder of Diversigen, a microbiome analysis service. NJA is Chief Scientific Officer at MicrobiomeDX, a microbiome analysis service. EH is Managing Editor at SynBioBeta.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

50. A study has shown that prolonged and repetitive exposure to *Porphyromonas gingivalis* increases the aggressiveness of oral cancer cells.

- a. True
- b. False

Prolonged and repetitive exposure to *Porphyromonas gingivalis* increases aggressiveness of oral cancer cells by promoting acquisition of cancer stem cell properties

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**Abstract** Periodontitis is the most common chronic inflammatory condition occurring in the human oral cavity, but our knowledge on its contribution to oral cancer is rather limited. To define crosstalk between chronic periodontitis and oral cancer, we investigated whether *Porphyromonas gingivalis*, a major pathogen of chronic periodontitis, plays a role in oral cancer progression. To mimic chronic irritation by *P. gingivalis* in the oral cavity, oral squamous cell carcinoma (OSCC) cells were infected with *P. gingivalis* twice a week for 5 weeks. Repeated infection of oral cancer cells by *P. gingivalis* resulted in morphological changes of host cancer cells

into an elongated shape, along with the decreased expression of epithelial cell markers, suggesting acquisition of an epithelial-to-mesenchymal transition (EMT) phenotype. The prolonged exposure to *P. gingivalis* also promoted migratory and invasive properties of OSCC cells and provided resistance against a chemotherapeutic agent, all of which are described as cellular characteristics undergoing EMT. Importantly, long-term infection by *P. gingivalis* induced an increase in the expression level of CD44 and CD133, well-known cancer stem cell markers, and promoted the tumorigenic properties of infected cancer cells compared to non-infected controls. Furthermore, increased invasiveness of *P. gingivalis*-infected OSCC cells was correlated with enhanced production of matrix metalloproteinase (MMP)-1 and MMP-10 that was stimulated by interleukin-8 (IL-8) release. This is the first report demonstrating that *P. gingivalis* can increase the aggressiveness of oral cancer cells via epithelial-mesenchymal transition-like changes and the acquisition of stemness, implicating *P. gingivalis* as a potential bacterial risk modifier.

**Keywords** *Porphyromonas gingivalis* .Oral cancer .Cancer stem cell .Interleukin-8 .Matrix metalloproteinase .Invasion

#### Introduction

It has been widely accepted that chronic inflammation predisposes an individual to carcinogenesis. For example, inflammatory bowel diseases and chronic viral hepatitis B or C are associated with colorectal cancer and hepatocellular cancer, respectively [1, 2]. Similarly, periodontitis, one of the most common chronic inflammatory diseases in the oral cavity, might act as a modifier of oral carcinogenesis and/or cancer progression. The incidence rate of chronic periodontitis increases with age. Similarly, the number of patients with oral cancer, mostly oral squamous cell carcinoma (OSCC), increases with age, usually

affecting people older than 40 years

[3]. Therefore, cancer cells found in these patients are likely to be surrounded by a tumor microenvironment infiltrated with chronic inflammatory cells and numerous pathogens, implying that a direct and constant influence from periodontitis could modulate oral cancer cells. Cohort studies reported significantly increased risk of oral and esophageal cancers in patients with tooth loss or periodontal disease [4–7],

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suggesting that chronic periodontitis may be one of the most important contributing factors to oral carcinogenesis and/or the biological behavior of oral cancers.

*Porphyromonas gingivalis*, an aerobic Gram-negative

bacteria, is frequently detected in patients with periodontitis and is widely accepted as a dominant causative factor responsible for age-

related chronic form of inflammation. The significance of *P. gingivalis* infection for the integrity of body organs and its pathological consequences have been investigated in recent years. These studies have provided insights into a potential association between *P. gingivalis* infection and systemic diseases with altered inflammatory or tissue degenerative processes, including atherosclerosis, low-birth weight complication in pregnancy, and diabetes [8]. Enhancement of inflammation by bacterial infections has been positively correlated with metastatic properties of mouse mammary carcinoma cells and human hepatocellular carcinoma [9, 10]. Therefore, it is possible that *P. gingivalis* may affect the prognosis of oral cancer patients by modulating the biological behavior of oral cancer.

Considering a tight association between inflammation and cancer, *P. gingivalis*-induced chronic periodontitis may also play a significant role in carcinogenesis as well. However, such a correlation between periodontitis and oral cancer is deduced only from epidemiological studies. Despite a few *in vitro* and *in vivo* studies to dissect the role of chronic periodontitis in the development and progression of oral cancer, conclusive experimental evidence to provide a direct link

between periodontitis and oral cancer is largely missing. Furthermore, our knowledge on the contribution of *P. gingivalis* to oral cancers is surprisingly scant. A recent study reported higher levels of antibodies against *P. gingivalis* linked to orodigestive cancer mortality [4], suggesting increased aggressiveness of orodigestive cancer associated with *P. gingivalis* infection. In this study, we aimed to investigate a potential link between periodontitis and oral cancer by creating an experimental condition that mimics a clinical course of *P. gingivalis*-induced chronic periodontitis in oral cancer cells. We have monitored changes in the invasive and tumorigenic properties, susceptibility to chemotherapeutic agents, and the acquisition of stemness in OSCC cells following repetitive *P. gingivalis* infection. We have

also defined important molecular signals mediating altered biologic behaviors of oral cancer cells induced by *P. gingivalis*.

#### Materials and methods

##### Bacteria and cell culture

*P. gingivalis* strain 381 was cultured anaerobically at 37 °C in trypticase soy broth supplemented with yeast extract (1 mg/ml), hemin (5 µg/ml), and menadione (1 µg/ml). Ca9

22 OSCC cells from Japanese Collection of Research (Biosources Cell Bank, Japan) were cultured in minimum essential medium (MEM; Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) at 37 °C with 5 % CO<sub>2</sub>. The Ca9-22 cells were treated with human recombinant interleukin-8 (IL-8 at 100 ng/ml; Peprotech, London, UK) at indicated time points.

##### Bacterial infection

OSCC cells were infected with bacteria at a multiplicity of infection (MOI) of 100 for 2 h in a CO<sub>2</sub> incubator and then washed with phosphate-buffered saline (PBS). In some experiments, the cells were infected with *P. gingivalis* and then treated with SB225002, an IL-8 receptor B antagonist, at 100 nM for 48 h to inhibit IL-8 signaling.

##### Carboxyfluorescein succinimidyl ester (CFSE) staining

A bacterial suspension was incubated with 10 µM CFSE (Molecular Probes, Eugene, Oregon, USA) in PBS for 15 min and then washed twice in PBS. After 2 h of infection with CFSE-stained *P. gingivalis*, the presence of the bacteria was observed with a confocal microscope (LSM510; Carl Zeiss, Germany).

##### Real-time PCR

Total RNA was extracted from Ca9-22 cells using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA quantity and purity were verified with a MicroQuant micro plate spectrophotometer (BioTek Instruments, Winooski, VT). For each sample, reverse transcription was performed at 42 °C for 15 min and 95 °C for 3 min, with 1 µg of total RNA using the QuantiTect reverse transcription kit (Qiagen). Real-time PCR assays were performed using the TOPreal SYBR Green PCR Kit (Enzynomics, South Korea) in the ABI 7500 Real-Time PCR Detection System (Applied Biosystems). The PCR program consisted of incubation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A negative control lacking cDNA template was included with each assay performed to assess the overall specificity. A standard melting curve was used to ensure amplification quality and specificity. The Ct value for each cDNA was then normalized to that of GAPDH used as a control. The real-time PCR primer sequences used include the bacterial 16S rRNA forward primer 5'-TCGGTAAGTCAGCG GTGAAAC, 16S rRNA reverse primer 3'-GCAAGCTGCC TTCGCAAT, IL-8 forward primer 5'-CTCTTGGCAGCCTT CCTGATT, IL-8 reverse primer 3'-TATGCACTGACATCTA AGTTCTTTAGCA, matrix metalloproteinase (MMP)-1 forward primer 5'-GGGAGATCATCGGGACA ACT, MMP-1 reverse primer 3'-AGCATCCCCTCCAATACCTG, MMP Tumor Biol.

10 forward primer 5'-CCTGGACCTGGGCTTTATGG, MMP-10 reverse primer 3'-CAGGGAGTGGCCAAGTTC AT, GAPDH forward primer 5-GAAGGTGAAGGTCGGA GTCAAC, and GAPDH reverse primer 3'-CAGAGTTAAA AGCAGCCCTGGT.

##### Immunofluorescence

Cells were fixed in 4 % paraformaldehyde for 10 min and permeabilized with 0.2 % Triton X-100 for 5 min. A primary antibody against  $\alpha$ -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was followed by an Alexa-Fluor 488-conjugated secondary antibody (Molecular Probes). Cell nuclei were visualized by staining with the DNA dye, DAPI (Vector laboratories, Birmingham, CA, USA). Images were obtained with a confocal microscope (Carl).

#### Western blot analysis

The presence of *P. gingivalis* in OSCC cells was confirmed using antibody from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa, USA). *P. gingivalis*-infected or non-infected cells were analyzed using antibodies against E- and N-cadherin (BD Biosciences, San Jose, CA, USA),  $\alpha$ smooth muscle actin, twist,  $\beta$ -actin (Santa Cruz Biotechnology), snail and slug (Cell Signaling Technology).

#### Migration and invasion assays

Cells in 200  $\mu$ l of medium containing 1% FBS were seeded on the top chamber (Transwell device, 8- $\mu$ m pore size, Costar, Cambridge, MA, USA), and 600  $\mu$ l of a cell-free medium containing 0.5  $\mu$ g/ml mitomycin (Sigma, St. Louis, MO, USA) and 10 % FBS was placed in the bottom chamber. For invasion assays, the upper surface of the membrane dividing two chambers was coated with 40  $\mu$ l of Matrigel (BD Biosciences). After 30 h of seeding, the cells that had migrated or invaded onto the lower surface of the membrane were stained with hematoxylin and eosin, mounted onto glass slides, and counted for their numbers.

#### MTT assay

Cells were seeded at a density of 8,000 cells per well in 96 well tissue culture clusters, infected with *P. gingivalis* at the MOI of 100 for 2 h on the next day, and then treated with taxol (Sigma) at indicated concentrations. Cells were further grown for 24 or 48 h before 200  $\mu$ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (500  $\mu$ g/ml) was added to each well to be metabolized for 4 h at 37 °C. The cells were washed twice with PBS, followed by the addition of 200  $\mu$ l of DMSO to dissolve formazan crystals. The absorbance of colored solution was quantified at 570 nm using a spectrophotometer (PerkinElmer, Santa Clara, CA, USA). Cell viability under each condition was determined as percent of the control value. Each condition was performed in triplicate wells, and data were obtained from at least three separate experiments.

#### Flow cytometry analysis

Cells were incubated with FITC-conjugated antibodies against CD44 (BD Pharmingen, Franklin Lakes, NJ, USA) and APC-conjugated CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 25 min at 4 °C in the dark. The stained cells were immediately analyzed using a flow cytometer (Beckman-Coulter Cytomics FC500, San Jose, CA, USA) equipped with an argon laser at the excitation wavelength of 488 and 633 nm. The results were based on the analysis of 20,000 cells.

#### Tumor sphere formation assays

Cells were seeded at a density of 1,000 cells per well in nonadherent 24-well culture plates that had been coated with a 10 % polyHEMA (Sigma) solution in absolute ethanol and dried overnight. After seeding, cells were incubated in a serum-free DMEM-F12 medium supplemented with 200 ng/ml of EGF (R&D Systems, Minneapolis, MN, USA), 20 ng/ml of basic FGF (Sigma), and B-27 supplement (Invitrogen, Carlsbad, CA, USA). After 5 days of an

incubation period, the number of spheroids per well was counted under a light microscope (Zeiss).

#### Multiplex bead (Luminex) assay

The levels of cytokines and MMPs in cell growth media were measured using the Milliplex Map Human cytokine/ chemokine kit and Milliplex Map Human MMP panel 2 kit (Millipore, Billerica, MA), respectively. Beads coupled with antibodies against interferon (IFN)- $\gamma$ , interleukins (IL) (1 $\beta$ , 8, and 10), tumor necrosis factor (TNF)- $\alpha$ , regulated on activation, normal T cell expressed and secreted (RANTES), vascular endothelial growth factor (VEGF), and MMPs (1, 2, 7, 9, and 10) were sonicated, mixed, and diluted in blocking buffer. The standard containing all cytokines or MMPs was prepared in blocking buffer. Each sample was then incubated with bead solution for 2 h at room temperature. Following a wash, each well was treated with primary detection antibody mixture diluted in blocking buffer for 1 h. After washing, streptavidin– phycoerythrin mixture was added to each well. The beads were resuspended in PBS. The Luminex 200 platform coupled with BioRad Bio-Plex software (BioRad, Hercules, CA) was used to measure the levels of cytokines and MMPs according to the manufacturers' protocols.

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##### Enzyme-linked immunosorbent assay (ELISA)

For detection of IL-8 in a cell culture supernatant, Enzyme Immunoassay kit (Enzo Life Sciences, Farmingdale, NY) and ELISA MAX™ Deluxe sets (Biolegend, San Diego, CA) were used according to the following kit's instructions. Standard curves were plotted as the control cytokine protein concentration (pg/ml) versus the corresponding mean optical density (O.D.) value of the replicates. The concentrations of putative IL-8-containing samples in duplicates were quantified based on the standard curve.

##### Silencing by siRNA

IL-8 siRNA (Bioneer Co. Ltd, Daejeon, Korea) is a 19-bp long double-stranded ribonucleotide with complementary sequence to human IL-8 mRNA. The sense sequence is CCAA GGC CAAG AGAAU AU. For silencing of IL-8, Ca9-22 cells were seeded and transfected with 100 nM of IL-8 siRNA using DharmaFECT transfection reagent (Dharmacon-Thermo Scientific, Waltham, MA). After a replacement of cell growth medium at 24 h, the effect of silencing was measured at 48 h following transfection of siRNAs.

##### Statistical analysis

Data were analyzed with SPSS (v21; Chicago, IL, USA) and GraphPad Prism (version 5.03; San Diego, CA, USA). The results from one representative experiment with triplicates were indicated as the mean  $\pm$  SD, unless otherwise indicated. Student's t test and one-way ANOVA with Tukey's multiple comparison method were used for comparison between two and multiple groups, respectively. P values less than 0.05 were considered statistically significant.

#### Results

*P. gingivalis* infection induces morphological changes in OSCC cells via an epithelial-mesenchymal-like transition

*P. gingivalis*, a major pathogen of chronic periodontitis, can play a crucial role in the progression of oral cancer, as *Helicobacter pylori* infection does in gastric cancer. We aimed to test this hypothesis by chronically infecting OSCC cells with *P. gingivalis*. We first identified

the internalization of *P. gingivalis* into OSCC cells after their co-culture for 2 h (Fig. 1a). To mimic chronic irritation caused by *P. gingivalis* in the oral cavity, OSCC cells were exposed to *P. gingivalis* twice a week for a 5-week period, with the confirmation of bacterial presence by a western blot analysis and real-time PCR (Fig. 1b,c). Short-term infection with *P. gingivalis* failed

to induce prominent changes in the shape of OSCC cells (data not shown). However, when repeatedly infected with *P. gingivalis*, the OSCC cells began to lose their characteristic polygonal shape and were eventually transformed into an extended, slender, and elongated morphology by the fifth week (Fig. 2a, b). To determine whether this morphological change of OSCC cells resembles epithelial-to-mesenchymal transition (EMT) at the molecular level, we monitored the levels of epithelial and mesenchymal markers in OSCC cells following *P. gingivalis* infection. Cytokeratin 13 (CK13), an epithelial marker dominantly expressed in normal oral lining mucosal cells [11], was significantly decreased after *P. gingivalis* infection. Furthermore, *P. gingivalis*-induced phenotypic changes

Fig. 1 *P. gingivalis* invades oral squamous cell carcinoma (OSCC) cells. (a) Ca9-22 OSCC cells were infected with carboxyfluorescein succinimidyl ester (CFSE)-stained *P. gingivalis* for 2 h and then observed under a fluorescent microscope. (b, c) For long-term exposure, cells were repeatedly infected with *P. gingivalis* twice a week for the indicated periods. The presence of *P. gingivalis* was confirmed by western blot analysis (b) and real-time PCR (c) Tumor Biol.

reflecting EMT were accompanied by upregulation of Ncadherin and  $\alpha$ -SMA, well-established mesenchymal markers, as well as transcriptional factors known to suppress epithelial cell characteristics, including snail, slug, and twist (Fig. 2c). Taken together, these results indicate that *P. gingivalis* is responsible for acquisition of the mesenchymal phenotype in OSCC cells.

*P. gingivalis* increases the migratory and invasive ability of oral cancer cells

Based on a well-established association between EMT and increased cellular motility and invasiveness, we examined whether oral cancer cells undergoing morphological changes following *P. gingivalis* infection could exhibit similar cellular behaviors. OSCC cells infected with *P. gingivalis* migrated to a significantly greater degree than uninfected control cells (Fig. 3a). In addition, infection with *P. gingivalis* significantly stimulated the invasion of OSCC cells through Matrigel, an

artificial barrier composed of endogenous basement membrane constituents, in contrast to little invasion observed in the uninfected control (Fig. 3b). Repetitive exposures of OSCC cells to *P. gingivalis* for a period of 5 weeks appeared to further facilitate their migratory and invasive ability (Fig. 3a, b). Taken together, these results further confirm the induction of EMT in OSCC cells upon chronic infection with *P. gingivalis*.

*P. gingivalis* slows proliferation of OSCC cells and confers resistance to a chemotherapeutic agent

In addition to tumor invasion and metastasis, EMT is also known to play important roles in cancer cell proliferation and its resistance to cell death [12, 13]. To examine whether *P. gingivalis*-induced EMT can modify the proliferative potential of OSCC cells, we performed an MTT assay in *P. gingivalis*-infected and non-infected OSCC cells. Starting in the first week, *P. gingivalis*-infected OSCC cells grew

Fig. 2 Long-term exposure to *P. gingivalis* accompanies EMT-like changes in the shape of OSCC cells. (a) Morphologic changes in *P. gingivalis*-infected OSCC cells were compared with non-infected control cells. Immunofluorescence staining against  $\alpha$ -tubulin was performed to define morphologic characteristics of *P. gingivalis*-infected OSCC cells. (b) Epithelial and mesenchymal markers of *P. gingivalis*-infected or non-infected cells were analyzed using western blot analysis

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slower than the non-infected control at both 24 and 48 h after seeding (Fig. 4a). When we analyzed the levels of cell cycle

related molecules, p21, a cell cycle inhibitor, was maintained at a higher level in *P. gingivalis*-infected OSCC cells

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compared to non-infected cells, while the level of Cyclin D1, one of the cell cycle progression molecules, was reduced (Fig. 4b). These findings suggest that proliferation of OSCC cells is suppressed via modulation of cell cycle-related

molecules by *P. gingivalis* and are consistent with a previous report demonstrating cell cycle arrest in the G0/G1 phase and lower

Fig. 3 *P. gingivalis* infection increases the migratory and invasive properties of OSCC cells.

(a, b) The migratory (a) and invasive potentials (b) of *P. gingivalis*-infected OSCC cells were compared with those of non-infected controls. All data are pooled from at least three independent experiments. Each bar represents the mean  $\pm$  standard deviation. Statistical significance was assessed by Student's t test. \* $P < 0.05$ , \*\* $P < 0.01$ , non-infected vs. *P. gingivalis*-infected OSCC cells

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Fig. 4 *P. gingivalis* infection inhibits the growth of OSCC cells and confers resistance to a chemotherapeutic agent. (a) Viability of non-infected or *P. gingivalis*-infected cells was analyzed using a MTT assay. (b) Expression levels of cell cycle-related molecules were measured with western blot analysis. (c) Non-infected or *P. gingivalis*-infected OSCC cells were treated

with taxol for 24 or 48 h, and their viability was then measured by a MTT assay. All data were calculated and plotted as the mean of at least three independent experiments. Each bar represents the mean  $\pm$  standard deviation. Statistical significance was assessed by Student's t test. \* $P < 0.05$ , \*\* $P < 0.01$ , non-infected vs. *P. gingivalis*-infected OSCC cells

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proliferative potential of the snail-overexpressing cells undergoing EMT [14]. Since cells at non-proliferative state are insensitive to a variety of cytotoxic drugs, *P. gingivalis*-infected OSCC cells were treated for 24 or 48 h with taxol, a classic chemotherapeutic reagent.

When compared to non-infected controls, *P. gingivalis*-infected OSCC cells showed reduced susceptibility to taxol, suggesting that *P. gingivalis* confer resistance to cytotoxicity caused by chemotherapeutic agents (Fig. 4c).

Cells infected with *P. gingivalis* exhibit cancer stem cell (CSC) properties

A growing body of experimental evidence has suggested that

EMT and drug resistance are associated with the acquisition of

CSC properties [15–17]. CSCs are thought to express cell surface markers such as CD44 and CD133 [18] that can be used in head and neck cancers as a representative indicator for cancer stemness [19, 20]. Importantly, the expression of both CD44 and CD133 was markedly increased upon chronic infection of OSCC cells with *P. gingivalis*, as evidenced by their signals

shifted toward the upper right quadrant in a flow cytometry analysis (Fig. 5a). This result suggests that *P. gingivalis* can contribute to the appearance of stemness in oral cancer cells. Another characteristic feature of CSCs is their ability to form cell spheres when cultured on non-adherent surfaces [18, 21]. Previous reports on oral cancers have demonstrated that CSCs expressing CD44 and CD133 are highly capable of forming spheres [20, 22].

Consistently, *P. gingivalis*-infected

Fig. 5 *P. gingivalis*-infected OSCC cells exhibit cancer stem cell properties. (a) Double immunofluorescence staining against CD44 and CD133 was performed to investigate cancer stemness of oral cancer cells. (b) Tumorigenic potential of *P. gingivalis*-infected OSCC cells was measured with a tumor sphere formation assay. Infected or non-infected cells were seeded on non-adherent culture plates and grown in serum-free medium for 5 days. The representative images from each condition were taken, and the number of spheroids per well was quantified (see "Materials and methods"). Each bar represents the mean  $\pm$  standard deviation. Statistical significance was assessed by Student's *t* test. \**P*<0.05, \*\**P*<0.01, non-infected vs. *P. gingivalis*-infected OSCC cells

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Fig. 6 *P. gingivalis* infection induces release of IL-8, MMP-1, and MMP-10 from host OSCC cells. (a) The level of IL-8 in the supernatants collected from *P. gingivalis*-infected OSCC cells was analyzed using a multiplex bead assay (top left) and ELISA (top right). The level of IL-8 mRNA was evaluated using realtime RT-PCR (bottom panel). (b, c) Expression levels of MMP-1 (b) and MMP-10 (c) were measured in *P. gingivalis*-infected cells. Cell culture supernatants of OSCC cells were harvested at the indicated time points from 1 to 7 days or 1 to 5 weeks following *P. gingivalis* infection. The amount of MMPs in the supernatants was then measured using a multiplex bead assay (top panel). The levels of MMP-1 and MMP-10 mRNA in OSCC cells were also measured using realtime RT-PCR (bottom panel). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison tests.

\**P*<0.05, \*\**P*<0.01, non-infected vs. *P. gingivalis*-infected OSCC cells

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OSCC cells with higher expression of CD44 and CD133 were far more potent in generating tumor spheres than uninfected controls (Fig. 5b, left). Importantly, the tendency to form tumor spheres was significantly promoted in OSCC cells after a chronic exposure to *P. gingivalis* beyond 3 weeks (Fig. 5b, right), further confirming the acquisition of cancer stemness by these oral cancer cells in response to their repetitive exposures to *P. gingivalis*. *P. gingivalis* increases release of IL-8, MMP-1, and MMP-10 from OSCC cells

A previous report has suggested that IL-8 plays an important role in induction of EMT and its maintenance [23], suggesting a potential link between cytokines and EMT processes. To elucidate whether similar molecular mechanisms drive the induction of EMT and acquisition of cancer stemness in *P. gingivalis*-infected OSCC cells, we monitored the release pattern of various cytokines. The expression levels of IFN- $\gamma$ , IL-1  $\beta$ , IL-8, IL-10, TNF- $\alpha$ , RANTES, and VEGF in cell culture supernatant were first analyzed with a multiplex bead assay at 1, 3, and 7 days post-infection with *P. gingivalis* (Fig. 6a, top left). Among an array of cytokines examined in the supernatant, a significant increase in their release was only observed for IL-8 and VEGF at 1 day postinfection (Table 1). Combined with a report of Fernando RI showing that IL-8 plays an important role in induction of EMT and its maintenance [22], we further observed the change of IL-8 using ELISA. A similar increase in IL-8 was also revealed with an independent ELISA analysis (Fig. 6a, top right). Such an

increase in IL-8 was correlated with a time-dependent increase in its mRNA (Fig. 6a, bottom), suggesting a transcriptional upregulation induced by *P. gingivalis* infection. In addition to cytokines, MMPs also play a crucial role in the process of invasion and metastasis of cancer cells. We investigated whether the increased invasiveness of *P. gingivalis*-infected OSCC cells was mediated via enhanced MMP activities. In contrast to prior studies demonstrating the importance of MMP-2 and MMP-9 in the invasive cancer cells [24], we found that MMP-1 and MMP-10 in OSCC cells were significantly upregulated by *P. gingivalis* infection in a time-dependent manner, with no significant increases in other MMPs (Table 2 and 3 and Fig. 6b, c). The most prominent change in MMP-1 and MMP-10 levels (13 and 27 times, respectively) was detected upon prolonged exposure to *P. gingivalis* for 5 weeks, suggesting that chronic irritation caused by *P. gingivalis* induces selective activation of MMPs in OSCCs to promote its invasive potential.

Invasive ability of *P. gingivalis*-infected OSCC cells is mediated via IL-8-induced increase in MMP-1 and MMP-10

Previous studies have suggested that cytokines can modulate the expression of MMPs that influences the progress and metastasis of tumor [25]. For instance, overexpression of IL-8 resulted in transcriptional upregulation of MMP-9, leading to increased invasiveness and tumorigenicity of prostate cancer cells [26]. Similar to these results, we found IL-8 dependent upregulation of MMPs, specifically MMP-1 and MMP-10, in OSCC cells chronically infected with *P. gingivalis*. IL-8 knockdown or blockade by a specific siRNA and SB225002, a receptor (CXCR2) inhibitor, respectively, led to decreased expression of MMP-1 and MMP-10 (Fig. 7a-d). To further establish a direct link between IL-8 and the invasion process of OSCC cells promoted by *P. gingivalis*, we examined the invasive ability of OSCC cells with reduced IL-8 expression. When IL-8 activity was blocked or downregulated, the invasiveness of *P. gingivalis*-infected OSCC cells was significantly attenuated (Fig. 7e).

Taken together, these data imply that IL-8 induced by *P. gingivalis* can promote invasive potential of OSCC cells via increased secretion of MMP-1 and MMP-10.

Table 2 MMP release from *P. gingivalis*-infected oral squamous carcinoma cells in a short period of time

MMPs (pg/ml) *P. gingivalis*

Control 1 day 3 days 7 days

MMP-1 3,374.5 5,294 7,609.5 7,695.5 MMP-2 70 70 70 70 MMP-7 183 165.5 165.5 148

MMP-9 2 5.5 5.5 4.5 MMP-10 595 1,313 1,380.5 1,233.5

Table 1 Cytokine release from *P. gingivalis*-infected oral squamous carcinoma cells

Cytokine (pg/ml) *P. gingivalis*

Control 1 day 3 days 7 days

GM-CSF 69.38 168 168 54.11 IFN- $\gamma$  0.57 0.94 0.84 <0.46 IL-10 <1.55 1.88 1.68 <1.55 IL-1 $\beta$

<1.01 <1.01 <1.01 <1.01 IL-6 2.59 14.37 4.91 2.51 IL-8 82.83 2,067 247 163 RANTES <2.12

3.38 2.92 <2.12 TNF- $\alpha$  <1.69 <1.69 1.96 <1.69 VEGF 219 757 1,277 291

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Discussion

Microbial infection has gradually been acknowledged as a major driver of inflammation-induced tumorigenesis in recent years. It appears that around 16 % of all cases of cancer worldwide can be associated with microbial infection [27, 28]. A contribution of viral infections to the development of malignant tumor has been confirmed by a number of

studies, but whether bacterial infections play a similar role remains unclear. The only established case of bacterial pathogens

Fig. 7 IL-8 release induced by *P. gingivalis* infection leads to upregulation of selective MMPs and promotion of invasive potential of oral cancer cells. (a,b) OSCC cells infected with *P. gingivalis* for 1 week were treated with an IL-8 receptor inhibitor (SB225002) for 48 h. The expression of MMP-1 (a) and MMP-10 (b) was assessed by a multiplex bead assay and real-time RT-PCR. (c, d) To confirm the effect of IL-8 on MMPs, *P. gingivalis*-infected OSCC cells were transfected with a siRNA construct against IL-8. The expression level of MMP-1 (c) and MMP-10 (d) after knockdown of IL-8 were assessed by a multiplex bead assay and real-time RT-PCR. (e) The degree of invasion through Matrigel-coated Transwell membranes by SB225002- or siRNA-treated OSCC cells was examined at 30 h after seeding. Statistical significance was assessed by Student's t test or one-way ANOVA with Tukey's multiple comparison tests. For Student's t test: \* $P < 0.05$  and \*\* $P < 0.01$ , control vs. indicated group. For one-way ANOVA: + $P < 0.05$  and ++ $P < 0.01$ , vehicle (DMSO) treated or *P. gingivalis*-infected (1 week) vs. indicated group, and ## $P < 0.01$ , scrambled siRNA-transfected or *P. gingivalis*-infected (1 week) vs. indicated group

Table 3 MMP release from *P. gingivalis*-infected oral squamous carcinoma cells in a long period of time

MMPs (pg/ml) *P. gingivalis*

Control 1 week 2 weeks 3 weeks 4 weeks 5 weeks

MMP-1 1,268 5,916 1,285 6,181 5,389 17,637 MMP-2 <7.88 232 68 232 232 232 MMP-7 <205.19 219 <205.19 <205.19 <205.19 236 MMP-9 14 15 17 50 30 40 MMP-10 62 726 265 563 546 1,682

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significantly associated with carcinogenesis is *H. pylori*, a causative factor in chronic gastritis, to aid the development of gastric cancer [29]. An increasing body of studies has recently suggested a possible link of *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Streptococcus bovis* infections to cervical, lung, and colonic cancers, respectively [30–32], albeit no solid conclusions have been drawn. Recent studies have demonstrated that microbial pathogens, including Epstein-Barr virus, hepatitis B and C virus, and *Citrobacter rodentium*, can play a role in the induction of EMT, just as *H. pylori*, a well-established EMT inducer in gastric epithelial cells [33–38]. In contrast, there is no study to define the role of *P. gingivalis* in EMT induction. Acquisition of a spindle-like morphology by repeated *P. gingivalis* infection in our study is consistent with *H. pylori*-induced morphological transformation characterized by an elongated cell shape with dramatic cytoskeletal rearrangements [39]. In addition, *P. gingivalis*-induced downregulation of epithelial cell markers with concomitant increases in mesenchymal markers implies that *P. gingivalis* can induce EMT-like changes in oral cancer cells. To our best knowledge, this is the first report to suggest *P. gingivalis* as a potent EMT inducer for oral cancer cells. EMT can also influence acquisition of cancer stemness that confers resistance to chemotherapeutic drugs. For instance, snail-induced EMT leads to a CSC-like phenotype associated with increased chemoresistance [12]. Prostate cancer stem cells with a slower proliferation rate exhibit survival advantage to a cytotoxic reagent [17]. However, our understanding about relationships among EMT, chemoresistance, and cancer stem cells is limited to a subset of cancers, mostly of viral origins [40,41]. The only known example demonstrating bacterial infections associated with

cancer stemness is upregulation of CD44 and expression of CSC properties induced by *H. pylori* infection in gastric epithelial cells [42]. Here, we demonstrate that repeated *P. gingivalis* infection induces EMT and develops resistance to taxol in host OSCC cells, along with acquisition of stemness indicated by increased expressions of both CD44 and CD133 and tumor sphereforming ability. Our results thus provide another example of bacterial infection that is tightly associated with EMT, chemoresistance, and cancer stemness. Epithelial cells undergoing EMT acquire the ability to migrate and invade, resulting in higher cancer aggressiveness. Thus, microbial pathogens capable of inducing EMT are likely to contribute to progression of infected host cancer cells. For instance, *H. pylori* infection leads to an increase in the invasiveness of gastric epithelial cancer cells [43–45]. Another example of increased invasiveness induced by a microorganism includes Epstein-Barr virus that promotes cell invasion *in vitro* via the synergistic actions of upregulated MMP-3 and MMP-9 [46]. In our study, *P. gingivalis*-infected OSCC cells showed increased expression of MMP-1 and MMP-10 via IL-8. It is an interesting parallel to previous studies reporting activation of MMP-2 or production of proMMP9 in oral epithelial or cancer cells following *P. gingivalis* infection [47–50]. Such subtype-specific activation of MMPs may stem from different host cancer cells, but the biologic significance of enhanced MMP-1 and MMP-10 in oral tumor progression may be more profound than other MMPs. Justilien et al. demonstrated that MMP-10 plays an important role in maintenance of CSCs and metastatic behavior [51]. *P. gingivalis* may accelerate the appearance of these stem cells as well as invasion of oral cancer cells via production of MMP-1 and MMP-10. Recently, it has been reported that a short exposure to *P. gingivalis* enhanced the invasive ability of SAS cells, another type of oral cancer cells with a higher invasive potential, but failed to promote invasiveness in less invasive Ca9-22 cells [52]. In our hands, Ca9-22 cells were transformed into highly invasive cells by the repetitive exposure to *P. gingivalis* over an extended period of time. As cells in the oral cavity are continuously exposed to and interact with microflora, repeated and prolonged exposure to *P. gingivalis* is likely to resemble a clinical condition encountered by old-aged patients with oral cancer and chronic periodontitis. Thus, our finding of transformed OSCC cells upon repetitive *P. gingivalis* infections supports the idea that chronic, but not acute, exposures to *P. gingivalis* can modify the biological behaviors of oral cancer cells and ultimately increase their aggressiveness, resulting in tumor progression. Here, we propose IL-8-dependent increases in MMP-1 and MMP-10 as a potential molecular mechanism underlying enhanced invasiveness. Considering the complexity of genetic and metabolic changes observed in cells undergoing EMT and acquiring cancer stemness, future studies will be focused on unveiling additional molecular players responsible for *P. gingivalis*-induced transformation of oral cancer cells. Outcomes of these studies will aid to develop new therapeutic remedies to improve the prognosis of oral cancer. In conclusion, we report, for the first time, that prolonged infection of oral cancer cells with *P. gingivalis*, more realistically reflecting the tumor microenvironment in oral cancer patients with chronic periodontitis, induces EMT-like changes and the emergence of CSC properties. Our results suggest that *P. gingivalis* is one of the most important risk modifiers that can transform oral cancer cells into more aggressive populations and shed light on the clinical significance of periodontitis to the progression and prognosis of oral cancer at the molecular level.

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