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Periodontal treatment and microbiome-targeted therapy in management of periodontitis-related nonalcoholic fatty liver disease with oral and gut dysbiosis

Microbiome-targeted therapy in periodontitis-related NAFLD

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Abstract

A growing body evidence from multiple angles proposes that periodontal disease, accompanied by oral inflammation and pathological changes in the microbiome, induces gut dysbiosis and is involved in the pathogenesis of Nonalcoholic fatty liver disease (NAFLD). A subgroup of NAFLD patients have a severely progressive form, namely nonalcoholic steatohepatitis (NASH), which is characterized by histological findings that include inflammatory cell infiltration and fibrosis. NASH has a high risk of further progression to cirrhosis and hepatocellular carcinoma. The oral microbiota may serve as an endogenous reservoir for gut microbiota, and transport of oral bacteria through the gastro-intestinal tract can sets up a gut microbiome dysbiosis. Gut dysbiosis increases the production of potential hepatotoxins, including LPS, ethanol, and other volatile organic compounds such as acetone, phenol and cyclopentane. Moreover, gut dysbiosis increases intestinal permeability by disrupting tight junctions in the intestinal wall, leading to enhanced translocation of these hepatotoxins and enteric bacteria into the liver through the portal circulation. In particular, many animal studies support that oral administration of *Porphyromonas gingivalis*, a typical periodontopathic bacterium, induces disturbances in glycolipid metabolism and inflammation in the liver with gut dysbiosis. NAFLD, also known as the hepatic phenotype of metabolic syndrome, is strongly associated with metabolic complications, such as obesity and diabetes. Periodontal disease also has a bidirectional relationship with metabolic syndrome, and both diseases may induce oral and gut microbiome dysbiosis with insulin resistance and systemic chronic inflammation cooperatively. In this review, we will describe the link between periodontal disease and NAFLD with a focus on basic, epidemiological, and clinical studies, and discuss potential mechanisms linking the two diseases and possible therapeutic approaches focused on the microbiome. In conclusion, it is presumed that the pathogenesis of NAFLD involves a complex crosstalk between periodontal disease, gut microbiota, and metabolic syndrome. Thus, the conventional periodontal treatment and novel microbiome-targeted therapies that include probiotics, prebiotics and bacteriocin would hold great promise for

preventing the onset and progression of NAFLD and subsequent complications in patients with periodontal disease.

Key Words: Periodontal disease; Nonalcoholic fatty liver disease; Microbiota; Dysbiosis; Metabolic syndrome; Probiotics

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Core Tip: A growing body evidence from multiple angles proposes that periodontal disease, accompanied by oral inflammation and pathological changes in the microbiome, induces gut dysbiosis and is involved in the pathogenesis of Nonalcoholic fatty liver disease (NAFLD). Thus, the conventional periodontal treatment and microbiome-targeted therapies that include probiotics, prebiotics and bacteriocin would hold great promise for preventing the onset and progression of NAFLD. In this review, we will describe the link between periodontal disease and NAFLD with a focus on basic, epidemiological, and clinical studies, and discuss potential mechanisms linking the two diseases and possible therapeutic approaches focused on the microbiome.

INTRODUCTION

Periodontal disease is an inflammatory disease that is induced by a structural and metabolic imbalance of the oral microbiome, namely an oral dysbiosis, and is characterized by significant periodontal tissue destruction and tooth loss[1-3]. Until recently, specific periodontopathic bacteria, such as the Red Complex, were thought to be the major pathogens of periodontal disease[4, 5], but the success of novel methods focused on the studying the oral microbiome with next-generation sequencing

approaches has revealed the possibility that a variety of previously unknown bacteria, fungi, and even viruses are associated with the periodontal disease[6-9].

Periodontal disease has also been reported to adversely affect the pathogenesis of various systemic diseases, including diabetes, coronary vascular disease, rheumatoid arthritis, and cancer[10,11]. As a major mechanism, it has been proposed that pro-inflammatory cytokines, periodontal pathogens, and their microbial components spread out from damaged periodontal tissues sites into the systemic circulation and thereby reach and affect other organ sites [1, 12-14]. Another idea has also emerged in the last decade that there is enteric translocation following the development of gut dysbiosis that originates from oral bacteria, and this is another pathway linking periodontitis and systemic disease[11]. Many studies have shown that swallowed periodontopathic bacteria can pass through the gastric acid barrier and reach the gut, thereby shifting the gut microbiome to an unhealthy state[15-21]. In addition, even oral commensal bacteria that are not normally pathogenic may proliferate and become established in the gut environment due to periodontal inflammation, and may even manifest pathogenic properties[22,23].

In the context of the association between periodontal disease and gut dysbiosis, nonalcoholic fatty liver disease (NAFLD) has received particular attention in recent years[11, 24] (**Figure 1**). NAFLD is currently the most prevalent chronic liver disease worldwide, with a marked increase in individuals with metabolic syndrome [25], and it is defined as a fatty liver condition diagnosed upon histological observation or image analysis, but other liver diseases/conditions, such as a history of alcohol consumption, viral hepatitis, and drug-induced liver injury are excluded[26, 27]. The majority of NAFLD patients (approximately 80%) have a good prognosis and simply have a fatty liver (NAFL), whereas the remaining 10-20% have a severely progressive form, namely nonalcoholic steatohepatitis (NASH)[27]. In addition to lipid deposition, NASH is characterized by histological findings that include inflammatory cell infiltration, ballooning degeneration and fibrosis of hepatocytes, and a reported high risk of further progression to end-stage liver disease, such as cirrhosis and hepatocellular carcinoma[28, 29]. NAFLD/NASH is considered a liver phenotype within various metabolic syndrome

components and has a robust and bidirectional association with metabolic complications such as diabetes, obesity, and cardiovascular disease[30].

Several animal studies have demonstrated that periodontopathic bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* cause increased insulin resistance and glucose intolerance through gut dysbiosis, as well as liver inflammation and fat deposition and fibrosis[20, 31-34]. These relationships between periodontal bacteria and NAFLD are also supported by epidemiological studies[31, 35-38], including cross-sectional and cohort studies, and further strengthened by clinical studies reporting that periodontal treatment improved NAFLD[37, 39].

The liver is in a unique anatomic location and as such it has a dual blood supply. The liver gets blood from the systemic circulation and the gut, with the portal vein responsible for most of this blood supply, and it transports nutrients, bacterial metabolites, toxins, and drugs absorbed from the gut to liver[40, 41]. In gut dysbiosis, abnormal growth and structural changes in intestinal bacteria result in an increase in hepatotoxic substances, such as endotoxin and ethanol, which reach the liver *via* the portal circulation[42-45]. In addition, increased intestinal permeability due to gut dysbiosis accelerates these hepatic exposures[46, 47].

Thus, management of the oral and gut microbiome may be an important preventive strategy for patients with periodontal disease and NAFLD (**Figure 2**). In this regard, besides traditional therapies, the use of adjunctive therapies that include probiotics and prebiotics may be beneficial in normalizing dysbiotic microbiomes present in periodontal tissues, the liver, and the gut. This review will describe the link between periodontal disease and NAFLD with a focus on basic, epidemiological, and clinical studies. Potential mechanisms linking the two diseases and possible therapeutic approaches focused on the microbiome will also be discussed.

2. PERIODONTAL DISEASE AS A RISK FACTOR FOR SYSTEMIC DISEASES

Periodontitis, a chronic inflammatory disease is characterized by a microbial dysbiosis and a progressive destruction of the tooth supporting structures[48]. In the US,

Periodontitis affects 42.2% of the population over the age of 30 and 59.8% over the age of 65[49]. Periodontitis is the major cause of tooth loss in adults, according to data from the World Health Organization,[50]. Periodontitis has a multifactorial pathogenesis that includes environmental, microbial, and host factors which affect disease outcomes. Several systemic diseases and conditions have been associated with periodontal disease, including cardiovascular disease, diabetes mellitus, and metabolic syndrome[11, 51-58].

The impact of systemic diseases and disorders on the periodontal tissues is well known, and the evidence indicates that periodontal disease may significantly enhance the risk for certain systemic diseases or conditions or alter their natural course[55, 59-63]. Many systemic conditions have been associated with periodontal diseases, although the level of evidence for each condition varies. Conditions for which the influence of periodontal disease is well documented include coronary heart disease (CHD) and related events, diabetes mellitus, preterm and low-birth-weight delivery, and preeclampsia; and respiratory conditions, such as chronic obstructive pulmonary disease[55, 59, 60]. A smaller but growing evidence base supports an association between poor oral health, tooth loss, or periodontitis and conditions, such as chronic kidney disease and renal insufficiency[64-69]; certain forms of cancer[70-74] affecting the liver, pancreas, and colorectal region; rheumatoid arthritis[75, 76] and altered cognitive function, dementia, and Alzheimer disease[24, 59, 77-81]. Here we will discuss the influence of periodontal disease in the context of diabetes and metabolic syndrome (**Figure 1**).

2-1. Diabetes

In terms of diabetes, a variety of studies have examined the effects of diabetes on the periodontal tissues, whereas others have examined the effect of periodontal disease on the control of diabetes[55]. A review of several studies including those with more than 22,000 patients found that the incidence of type 2 diabetes (i.e., new diagnoses) was significantly greater in individuals with periodontal disease than in those without periodontal disease[82]. Severe periodontal disease was associated with a significant worsening of glycemic control over time in patients with type 2 diabetes[83]. Those with

severe periodontitis at baseline had a greater incidence of worsening glycemic control over a 2- to 4-year period as compared with those without periodontitis at baseline. Furthermore, periodontitis was known to have preceded the worsening of glycemic control in this study. In addition, numerous systematic reviews and meta-analyses have consistently shown that periodontal therapy is associated with a significant and clinical improvement in glycemic control in patients with diabetes and periodontitis[84-86]. Furthermore, meta-analyses also indicate a higher prevalence and severity of periodontal disease in diabetic patients and vice versa[87, 88].

Understanding the mechanisms that underlie other infections is helpful to understanding the mechanisms whereby periodontitis influences glycemic control. Systemic inflammation is well known to play a major role in insulin sensitivity and glucose dynamics. Periodontitis can trigger or sustain an elevated systemic chronic inflammatory state, as shown by increased levels of serum CRP, IL-6, and fibrinogen in individuals with periodontal disease[83, 89, 90]. Inflammation triggers insulin resistance, which often accompanies systemic infections. For example, acute viral and bacterial infections can increase insulin resistance and aggravate glycemic control[91, 92]. Systemic infections increase insulin resistance through several mechanisms. The insulin resistance, in turn, prevents glucose from entering target cells, causing elevated blood glucose levels. The elevated blood glucose then requires increased pancreatic insulin production to maintain normal glycemic levels. The insulin resistance can persist for weeks or months after the individual has recovered from the illness. Individuals with type 2 diabetes who already have significant insulin resistance, may experience additional tissue resistance to insulin as a result of infection, and thus exhibit an exacerbated poor glycemic control. In addition, studies suggest a relationship between the inflammatory status associated with diabetes or metabolic syndrome and the inflammatory status of periodontal disease[93, 94]. Indeed chronic tissue inflammation has been recognized in the onset of metabolic diseases[95].

Chronic gram-negative periodontal infections may also lead to increased insulin resistance and poor glycemic control[96]. In individuals with periodontal disease, persistent systemic exposure to periodontopathic bacteria and their products results in

an up-regulation of the immunoinflammatory response, with elevation in serum levels of proinflammatory mediators, such as IL-1β, TNF-α, and IL-6, similar to well-recognized systemic infections, but on a more persistent, chronic basis. Increased serum levels of several cytokines, including TNF-α and IL-6, are associated with increased insulin resistance. These processes would help explain the worsening glycemic control associated with severe periodontal disease. Periodontal treatment, which is meant to decrease the bacterial insult and reduce inflammation, may lead to decreased systemic inflammation that may restore insulin sensitivity over time, and thereby result in improved metabolic control. This hypothesis would be supported by the many studies showing the improved glycemic control following periodontal therapy.

Much focus has been placed on the fact that chronic conditions can change the microbiome. As an example, individuals exhibiting obesity or type 2 diabetes demonstrate a modified gastrointestinal microbiome (reviewed by [97-100]). In addition, rodents exposed to *Enterobacter cloacae* B29, an intestinal microbe obtained from obese subjects with diabetes, develop insulin resistance and obesity[101]; indicating that bacteria can also directly induce diabetes-related symptoms. Also, modulating the microbial-mediated mucosal immunity and inflammation may help attenuate type 2 diabetes; by mechanisms that include modulation of T lymphocytes[102],[103]. Since there are associations between type 2 diabetes, periodontal disease, and changes in the gut microbiome of individuals with type 2 diabetes, additional focus has been placed on microbiome changes in the periodontal tissues of individuals with type 2 diabetes.

Studies have shown that type 2 diabetes alters the subgingival and salivary microbial profile by lowering richness and diversity[104-109]. These findings support the observations found in the gut microbiome[97-100, 110]. Studies show that the subgingival microbiome diversity is not only decreased in individuals with type 2 diabetes, but when separated by adequate or inadequate glycemic control, there is a further decrease in the microbiome diversity in those with inadequate glycemic control (hemoglobin A1c ≥8)[111]. However, another study which assessed the microbiome present in the saliva of 17 subjects with periodontal disease and type 2 diabetes was not able to find a relationship between

glycemic control and changes in the oral microbiota. Although, the study found that in individuals with type 2 diabetes, the microbial composition varied significantly between obese (BMI ≥30) and non-obese individuals with type 2 diabetes, such that obesity reduced the oral microbial diversity[112]. Furthermore, although the subgingival and supragingival microbial diversity decreases when subjects with type 2 diabetes are compared to normoglycemic individuals, the microbial shift is less in individuals with periodontal disease with type 2 diabetes subjects than in normoglycemic individuals[105, 106, 113].

In summary, the subgingival and salivary microbial profile is altered by type 2 diabetes by decreasing microbial diversity and richness (**Figure 1**). In terms of glycemic control, there is a further decrease in microbial diversity when there is inadequate glycemic control. Furthermore, the microbial shift observed in individuals with periodontal disease is less prominent in individuals with type 2 diabetes compared to normoglycemic controls. In addition, microbial diversity is further reduced in smokers. Additional research is needed to examine a potential two-way relationship between diabetes and the periodontal microbiome; namely that periodontal microbes may directly induce diabetes-related pathology, as data indicate that microbes derived the gastrointestinal tract of obese patients with diabetes can themselves mediate symptoms related to diabetes in animal models.

2-2. Metabolic syndrome

We will next discuss the association between metabolic syndrome and periodontal disease. Metabolic syndrome is a characterized by constellation of conditions that elevate the risk for cardiovascular disease and double the risk for type 2 diabetes [114-117]. Approximately 34% of the US population[118] and 10% of US adolescents are affected by Metabolic syndrome[119]. Metabolic syndrome prevalence increases with age and varies with ethnicity and gender[120]. Metabolic syndrome is defined slightly differently by different agencies. That conveyed by the National Cholesterol Education Program Adult Treatment Panel III is the most frequently used definition; an individual has to exhibit at

a minimum, three out of the five risk factors: 1) low plasma levels of HDL cholesterol, 2) increased values for plasma triglycerides, 3) increased abdominal circumference, 4) elevated blood pressure, and 5) elevated glucose levels[121]. Pre-diabetes is included in metabolic syndrome as it presents with insulin resistance and is highly predictive of new-onset type 2 diabetes[122].

Abdominal obesity and insulin resistance are primary risk factors for metabolic syndrome are. Physical inactivity, aging, and hormonal imbalance also contribute to metabolic syndrome[123]. A primary trigger and risk factor for the majority of mechanisms in metabolic syndrome is visceral adiposity [124]. Precise processes that underlie the systemic response seen in metabolic syndrome have not been well elucidated, but data highlight that the inflammatory response present this disease mediates endothelial dysfunction that may, in turn, promote type 2 diabetes and cardiovascular disease [125-127]. There has been a significant interest in the association between metabolic syndrome and periodontal disease, since both are characterized by insulin resistance and systemic inflammation[128, 129]; common pathways whereby they could impact each other. Cross-sectional, longitudinal, and meta-analyses studies have evaluated the association between metabolic syndrome and periodontal disease. Most data indicates that there is an association between metabolic syndrome and periodontal disease[57, 58, 130-156]. Three meta-analyses, discussed here, found an association between periodontal disease and metabolic syndrome (odds ratio from 1.38 to 1.99)[133, 134, 157]. One meta-analysis [157] that discussed 39 studies showed an association between metabolic syndrome and periodontal disease (crude odds ratio of 1.99 (95%CI: 1.75-2.25) and an adjusted odds ratio of 1.46 (95%CI: 1.31-1.61)). A subgroup analysis focused on different countries was also included in this study and the results showed a combined odds ratio of 1.75 (95%CI: 1.31-2.34) for the United States; 1.68 (95%CI:1.41-2) for Japan; 1.81 (95%CI: 1.35-2.42) for Korea, and 2.29 (95%CI: 1.53-3.41) for China[157]. Another meta-analysis[134] of 26 manuscripts that included radiographic and clinical exam data reported an association between these two diseases (odds ratio of 1.38 (95%CI: 1.26-1.51)). This suggested that patients with metabolic syndrome are 38% more likely to exhibit periodontal disease[134].

A systematic review/meta-analysis of multiple manuscripts that reported on 36,337 individuals, found a positive association between periodontal disease and metabolic syndrome (odds ratio of 1.71 (95%CI: 1.42-2.03))[133]. A comprehensive review further found a positive association between metabolic syndrome and periodontal disease[158]. Furthermore, various animal studies that employed different periodontal disease models demonstrated that rodents with metabolic syndrome or obesity due to a high-fat diet, also exhibited exacerbated periodontal bone loss[159-163].

In terms of assessing metabolic syndrome's role in contributing to periodontal disease, two longitudinal studies found that metabolic syndrome increases the risk of periodontal disease development and progression[137, 144]. Specifically, one study found that individuals with metabolic syndrome were 2.6 times more likely to develop periodontal disease. Furthermore, with increased attributes of metabolic syndrome, the periodontal disease exhibits a higher prevalence and a more extensive presentation [137, 140]. Even though most studies found an association between periodontal disease and metabolic syndrome, some reports found a minimal association for these diseases[136, 147, 153, 164-166]. However, most of the studies that found no association were either cross-sectional or shorter longitudinal studies (only one year), and one study was performed on a young cohort of subjects[136], that had low levels of both metabolic syndrome and periodontal disease. Furthermore, in longitudinal study of three years, researchers found that toothbrushing frequency was inversely related to the incidence of metabolic syndrome[167].

Most studies have concluded that periodontal disease may contribute to the development or exacerbation of metabolic syndrome[57, 142, 168]. In a cross-sectional study of 190 individuals, researchers found that periodontal disease and periodontal bone loss may contribute to the development of metabolic syndrome[57]. A four-year longitudinal study of 1,023 adults found deeper periodontal pockets associated with a positive conversion of metabolic components (odds ratio 1.6; 95%CI: 1.1-2.2)[142]. Furthermore, another study reported that in individuals with metabolic syndrome a decrease in periodontal inflammation reduced C-reactive protein levels in those subjects[168].

There has been an increasing interest in the role of the microbiome in metabolic diseases and disturbances. Metabolic diseases change the gut microbiome (reviewed by [100]), and the oral microbiome varies significantly between individuals with a healthy periodontium and those with periodontal disease[169]. Furthermore, changes in the gastrointestinal microbiome are associated with metabolic syndrome and obesity[170]. Obesity affects the oral microbiome in the context of type 2 diabetes[112], plus it lowers the diversity of the microbiome within the distal gut[171, 172]. Further, subjects with decreased microbial diversity exhibit significantly more insulin resistance, adiposity, and dyslipidemia in contrast to individuals with elevated microbial richness[172]. A study focused on 17 subjects with type 2 diabetes and with advanced periodontal disease found that the composition of the oral microbiome varied significantly between non-obese and obese (BMI ≥30) subjects. Those findings suggested that obesity is related to a decreased oral microbial diversity [112]. Our research group also showed that the oral microbiome is altered in individuals with metabolic syndrome as compared to healthy individuals[173]. In addition, there are a growing number of studies suggesting potential links between the gut or oral microbiome and obesity[174-176].

In vitro and *in vivo* (animal) studies have shown how dyslipidemia (high-fat diet) compounds the effects of metabolic syndrome on periodontitis[159-163, 177], and a variety of studies have assessed the role of impaired glucose in periodontal disease. A study on obese mice (metabolic syndrome model) inoculated with *P. gingivalis* and fed a high-fat diet that did not exhibit diabetes, revealed that these mice had 40% more alveolar bone loss and higher levels of *P. gingivalis* versus control mice [159]. Using the same model, another group of researchers found that the high-fat diet group exhibited metabolic syndrome, including dyslipidemia, obesity, hyperinsulinemia, and insulin-resistance. Further, the group with metabolic syndrome exhibited significantly higher osteoclast formation and alveolar bone loss. Similarly, our previous study showed that the ligature-induced periodontitis with *P. gingivalis* infection in rats fed a high-fat diet not only exacerbated the alveolar bone resorption, but also induced increase in levels of fasting blood glucose and liver damage markers, and systemic inflammation[162, 163].

Moreover, lipopolysaccharide-induced-periodontitis exacerbated inflammatory cytokine expression (interleukin-6, MCP-1, RANK-L, and MCSF), osteoclast formation, and alveolar bone loss[161]. Studies that used osteoblasts from obese mice showed a reduction in cell proliferation and an elevated osteoblast cell death following *P. gingivalis* treatment versus controls [178]. Furthermore, obese rodents with metabolic syndrome exhibited significant elevated levels of *A.actinomycetemcomitans*-lipopolysaccharide-induced alveoalr bone loss verus the non-obese, non-metabolic syndrome animals. In addition, a cholesterol-lowering medication frequently given to patients with metabolic syndrome[179], attenuated alveolar bone loss in both groups, further supporting the role of dyslipidemia in periodontal inflammation[180]. In this regard, our research found that submucosal administration of radio-labeled *P. gingivalis* LPS to the palatal gingiva of high-fat diet-induced obese rats led to a significant accumulation of LPS in the liver and gingiva compared to other organs, including the spleen, kidney, and brain. Furthermore, the gingiva and fatty liver of obese rats demonstrated an increased level of *P. gingivalis* LPS and a prolonged accumulation time compared to mice fed a normal diet. In addition, a high-fat diet further delayed the metabolic clearance of *P. gingivalis* lipopolysaccharide from the gingiva and liver[181].

The role of lipids in periodontal disease was further explored using *in vitro studies*, and these studies showed that fatty acids augmented the lipopolysaccharide-mediated expression of markers involved in periodontal disease, for example interleukin1-a, interleukin1-b, CXCL10, CD86, CSF2, MCP1, TLR2, TNFa, and CD14 [161]. Further *in vitro* studies performed on macrophages demonstrated a significant increase in CD36, a major fatty acid receptor, upon treatment with lipopolysaccharide plus palmitate in comparison to macrophages only treated with lipopolysaccharide or palmitate alone[177]. Periodontal disease and metabolic syndrome, independently and significantly increased the levels of CD36, and when metabolic syndrome and periodontal disease were present simultaneously, there was an additive effect. The expression of CD36 in periodontal tissues also positively correlated with osteoclast formation.

Taken in aggregate, human studies and animal models show an association between metabolic syndrome and periodontal disease, and metabolic syndrome can change the oral microbiome and potentiate the harmful effects of periodontal disease.

3. RELATIONSHIP BETWEEN PERIODONTAL DISEASE AND NAFLD

Periodontal disease has been associated with liver diseases, such as NAFLD, liver cirrhosis, and hepatocellular carcinoma, and even a possible worse prognosis for liver transplantation **(Figure 1)**. The relationship between periodontitis and NAFLD is supported by a number of epidemiological studies[31, 35-37, 182-200]. Cross-sectional studies have also shown that the severity of periodontal disease correlates significantly with levels of blood liver injury markers (e.g.: alanine aminotransferase: ALT, aspartate aminotransferase: AST, and r-GTP)[182, 183, 185], hepatic imaging by ultrasound and CT, and a formula-based scoring system for estimating the degree of fatty liver and liver fibrosis [184, 191, 196]. Furthermore, several cohort studies have strengthened the causal relationship between periodontal disease and NAFLD. Akinkugbe *et al* followed 2,623 non-NAFLD subjects for more than 5 years (mean 7.7 years) in a health survey conducted in West Pomerania in Germany, and found that a history of periodontitis was an independent risk factor contributing to the development of NAFLD, which was diagnosed by abdominal ultrasonography and serum ALT levels[189]. Compared to subjects without CAL ≥3 mm, the incidence of NAFLD was significantly higher in patients with <30% or ≥30% affected sites with CAL ≥3 mm. The adjusted incidence rate ratio for NAFLD was 1.28 for <30% and 1.60 for ≥30% of affected sites with a statistically significant difference, respectively. Similarly, the incidence rate difference was statistically significant at 5.49 for < 30% of sites and 11.11 for ≧30% of sites. Helenius-Hietala *et al* found in a 13-year cohort study of 6,165 subjects participating in a Finnish population-based health study, the proportion of sites with deep periodontal pockets positively correlated with the hazard ratio for developing severe hepatic diseases[35]. In addition, the hazard ratio was not correlated with periodontitis in those who were non-NAFLD at the study entry, whereas was 6.94 in those who were developed NAFLD with

significant correlation. Based on systematic reviews and meta-analyses, this accumulating evidence highlights the potential for periodontal disease as a direct and indirect risk factor for the development and progression of NAFLD.

Periodontal disease has been reported to exacerbate metabolic diseases including diabetes, dyslipidemia and obesity through disturbance of energy homeostasis, and given the close association between NAFLD and metabolic syndrome, this underscores the importance of understanding the periodontal-metabolic relationship in the NAFLD pathogenesis. Two-thirds of patients with obesity and type 2 diabetes have been found to have fatty liver, and both excessive body mass index and visceral obesity are recognized as risk factors for NAFLD[201, 202]. Metabolic syndrome is also related to an increased risk of more progressive liver disease, such as NASH, cirrhosis, and future liver failure, in patients with NAFLD[203-205]. A 5 year-followup cohort study reported by Kuroe *et al* [200] demonstrated that in 341 Japanese subjects with nonalcoholic fatty liver (NAFL) without complications of liver fibrosis, stratified analysis by obesity showed that in obese subjects, severity of periodontal destruction was significantly correlated with liver fibrosis with an odds ratio of 2.87, whereas in non-obese subjects they were not correlated. Moreover, in metabolic diseases animal models induced by high-fat diet or high-cholesterol diet, the ligature-induced experimental periodontitis and periodontopathic bacteria infection exacerbated disturbances of glycolipid-metabolism in the liver and enhance the NAFLD progression[162, 206, 207]. Although not all mechanisms explaining the interaction between periodontal disease and NAFLD *via* metabolic diseases are known, diffusion of inflammatory mediators and periodontopathic bacteria and their components, and release of reactive oxygen species from inflamed periodontal tissues into the circulation can mediate low-glade systemic inflammation and, thereby exacerbate insulin resistance in obesity and diabetes[208].

Data over the last 10 years strongly suggest that *P. gingivalis* is involved in NAFLD and NASH. *P. gingivalis* has many virulence factors, such as collagenase, trypsin-like gingipain enzymes, LPS and fimbriae, that are also known to trigger intracellular signaling events[209]. Yoneda *et al*[37] analyzed various periodontopathic bacteria in saliva

collected from non-NAFLD control subjects and NAFLD patients using PCR assays and showed that the detection frequency of *P. gingivalis* was significantly higher in the NAFLD patients. In addition, injection of *P. gingivalis* via the jugular vein dramatically accelerated NAFLD progression in mice fed a high fat diet. We have previously reported that *P. gingivalis* infection combined with ligature-induced periodontitis increased serum ALT and LPS levels and hepatic fat deposition in high-fat diet-induced obese rats with insulin resistance[162, 163]. Conversely, hyperglycemia can promote translocation of *P. gingivalis* from the oral cavity to the liver and reduce hepatic insulin-induced glycogen biosynthesis in mice[210]. For other periodontopathic bacteria, enteral infection with *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* as well as *P.gingivalis* may contribute to NAFLD by altering the gut bacterial flora and metabolome in mice[31, 33].

4. MECHANISMS BY WHICH PERIODONTAL DISEASE EXACERBATES NAFLD BASED ON THE ORAL-GUT-LIVER AXIS

At the present time, dual pathways have been proposed to explain the connection between periodontal disease and liver pathology, namely hematogenous and enteral diffusion of hepatotoxic components.[11]. The liver is the largest parenchymal organ and, due to its unique anatomical location, it has a dual blood supply coming from the systemic circulation and the gastrointestinal tract[40], which helps explain how toxic factors diffuse from periodontal tissue and reach the liver. Eighty % of the total blood supply received by the liver is derived from the enteric portal vein, which is rich in nutrients, bacterial metabolites, and food antigens. The remaining 20% is supplied by the hepatic artery, which is the nutrient vessel of the liver and branches off the abdominal aorta. Thus, the liver is the hemodynamic confluence in the human body, and its unique characteristics allow for the maintenance of a diverse intrahepatic cell population composed of metabolically active hepatic parenchymal cells, connective tissue cells, and various immune cells[41].

One of the pathways linking the oral cavity and liver is thought to be hematogenous diffusion, which is caused by inflammatory mediators and microorganisms passing through the systemic circulation due to hyperpermeability of the pocket epithelium and micro-ulceration in the periodontal inflamed tissue[1, 12]. For example, the serum levels of reactive oxygen species and inflammatory cytokines are elevated in periodontitis patients, suggesting a mild chronic inflammatory state systemically[211, 212]. In fact, epidemiological studies have shown that Elevated blood level of C-reactive protein (CRP), which is secreted by hepatocytes upon stimulation of proinflammatory cytokines, are a common modifier of periodontitis and NAFLD[188, 192]. With regard to microbiological invasion, mechanical injuries such as daily oral hygiene activities, chewing movements, and calculus removal as periodontal treatment have been reported to increase the frequency of bacteremia and endotoxemia in patients with periodontal disease compared to healthy subjects[213, 214].

Another possible pathway linking the oral cavity to the liver is the transport of oral bacteria through the gastrointestinal tract, resulting in abnormalities in the gut microbiota[43, 215, 216]. Patients with periodontitis have a characteristic oral microbiome, and they continuously and unknowingly swallow pathogens present in saliva and dental plaque[21]. A part of oral bacteria can reach the intestinal tract, even in systemically healthy individuals and regardless of the harsh acidic environment in the stomach. Thus, the oral microbiome may function as an endogenous reservoir that supply novel bacteria to the gut microbiome[17]. It is widely known that gut dysbiosis is closely associated with the pathogenesis of NAFLD through changes in gut bacterial composition and metabolites[215, 217, 218]. In a state of gut dysbiosis, the production of choline which is an essential substance for lipolysis is decreased, whereas LPS, ethanol and volatile organic compounds (e.g. acetone, phenol and cyclopentane) are increased, which are potential hepatotoxin[42, 43, 45]. Moreover, the gut dysbiosis enhanced intestinal permeability by disrupting intercellular junctions in the intestinal mucosa, leading to increased translocation of enteric bacteria and their metabolites to the liver. Therefore, the liver is

constantly exposed to various substances of intestinal derivation, which diffuse into the liver through the enterohepatic portal circulation.

A study by Lourenço *et al*[21] reported that numerous oral taxa related to periodontal inflammation and destruction were detected in the gut microbiome of individuals, regardless of periodontal condition. Patients with periodontal disease were characterized by a higher ratio of Firmicutes/Bacteroidetes, an enrichment in Euryarcheota, Verrucomicrobiota, and Proteobacteria, and a less diverse gut microbiota than those with healthy periodontal status. Kawamoto *et al*[16] found that fecal samples from patients with severe periodontitis were enriched in *Acidaminococcus, Clostridium, Lactobacillus, Bifidobacterium, Megasphaera*, and *Romboutsia*compared to those from healthy subjects. However, few studies have observed changes in the gut microbiota in patients with periodontal disease, and consistent trends in the gut dysbiosis remain unclear, with wide variations. In the future, it would be interesting to compare the modifications of the gut microbiota in the state of periodontal disease with those observed in NAFLD, diabetes, and obesity to elucidate the complex relationship between periodontal disease and systemic diseases.

Many animal studies have shown that orally administration of periodontal pathogens caused insulin resistance and hepatic fat deposition in rat and mice, accompanied by alterations in gut microbiota and glycolipid-metabolism[19, 20, 31-33]. In particular, *P. gingivalis,* known as a keystone pathogen, causes mucosal inflammation *via* contributing to oral and gut microbiome dysbiosis[20, 219]. Yamazaki *et al*[33] showed that in an NAFLD mouse model fed a high-fat diet, oral administration of *P. gingivalis* or *P. intermedia* changed the composition of gut microbiota and blood metabolome, resulting in a shift in liver transcriptome expression toward the NAFLD pathogenic form. In contrast, application of commensal oral bacteria *Actinomyces naeslundii* and *Veillonella rogosae* did not affect NAFLD progression. Komazaki *et al*[31] reported that oral infection with *A.actinomycetemcomitans* may contribute to NAFLD by altering the bacterial flora and glucose metabolism in mice. This result is also consistent with the fact that Anti-*A.*

actinomycetemcomitans antibodies were positively correlated with visceral fat area, insulin resistance, and serum AST level in 52 Japanese NAFLD patients.

Recent studies have revealed that inflammation of periodontal tissue alters gut bacteria and metabolites even without specific periodontopathic bacterial infections [23, 220, 221]. Even commensal oral bacteria, which are not notably pathogenic in the oral cavity, such as *Enterobacter spp.* and *Klebsiella spp.* when stimulated to proliferate by ligature-induced periodontitis, can mediate ectopic intestinal colonization and may play an important role in the exacerbation of enteritis[23]. Therefore, future studies on the pathogenesis of NAFLD should not only focus on individual periodontal pathogens, but also comprehensively evaluate the relationship between changes in the oral and gut microbiota as a whole.

5. PERIODONTAL DISEASE FROM THE VIEWPOINT OF ORAL DYSBIOSIS

Polymicrobial diseases represent clinical and pathological conditions caused by 2 or more microorganisms[222]. The bacterial microbiome in the oral cavity is composed of more than 700 bacterial species[223] and is associated with the development and progression of oral diseases[224]. Periodontal diseases are representative oral polymicrobial diseases that involve a microbiome imbalance known as dysbiosis[225, 226], leading to dysregulated host-microbial crosstalk that induces periodontal inflammation and alveolar bone loss[227]. The 2009–2010 and 2011–2012 cycles of the National Health and Nutrition Examination Survey reported that almost 50% of adults aged ≥30 years in the United States were affected by periodontitis[228]. In addition, periodontitis is associated with systemic diseases, such as type 2 diabetes, obesity, cardiovascular disease, preterm low birth weight, and nonalcoholic fatty liver disease[31, 229-233]. Therefore, a complete understanding of periodontal disease is essential to prevent a decline in quality of life.

The current theory suggests that gingival lesions are precursors of periodontitis[234]. Löe *et al* demonstrated that the accumulation of bacterial plaque causes gingivitis in humans, and gingival inflammation is resolved by the removal of the plaque[235]. In addition, continued plaque accumulation in animal models induced gingivitis and gradually

developed into periodontitis[236]. These results clearly indicate that bacterial plaque is involved in periodontitis. This belief was called the "nonspecific plaque hypothesis" which states that a sufficient accumulation of microorganisms results in destructive inflammation in periodontal tissues[237]. The contradiction between the nonspecific plaque hypothesis and actual clinical findings led scientists to propose the "specific plaque hypothesis". This hypothesis was that specific bacterial species were involved in the etiology of periodontitis[238]. However, potential periodontal pathogens could not be identified because approximately 30–100 bacterial species inhabit the periodontal pocket, and plenty of other bacterial species may have been unculturable[239].

Although culture methods have traditionally been useful for the identification of causative bacteria, DNA-based bacterial detection methods using molecular biology techniques have also contributed to the characterization of oral bacterial composition. Socransky *et al* reported on findings relating to the specific plaque hypothesis where they targeted 40 subgingival bacterial species using the checkerboard DNA-DNA hybridization method. These bacteria were mainly divided into five groups: red, orange, green, yellow, and purple complexes. *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola* were defined as periodontal pathogens and classified as the red complex because they exhibited a strong relationship with probing pocket depth and bleeding on probing. The orange complex was also associated with probing pocket depth and appeared to be closely related to the red complex[4]. Red and orange complexes were detected more frequently in patients with periodontitis than in healthy subjects[240]. Periodontitis has been reported to be associated with many systemic diseases[241], especially diabetes caused by metabolic syndrome. The prevalence of periodontitis in diabetic subjects was higher than that in non-diabetic subjects[242]. In addition, poorly controlled diabetic subjects have more severe periodontitis[243]. In periodontitis sites, a higher quantity of *P. gingivalis* in poor controlled diabetic subjects was observed than that of non-diabetic subjects[244]. Haffajee *et al* reported that a reduction in the red complex was observed at sites where periodontal treatment with scaling and root planning alone or with systemically administered azithromycin, metronidazole, or a sub-antimicrobial

dose of doxycycline was performed[245]. In another study, Duarte *et al* also found a reduction in red and orange complexes after periodontal treatment[246]. Therefore, members of the red complex are still considered periodontal pathogens; however, some inconsistency exists in this concept. Previous studies demonstrated that *Streptococcus mutans* and *Streptococcus salivarius*, which were not defined as periodontal pathogens in the complex-based classification, causing attachment loss of periodontal tissues in animal studies[247, 248]. Another example of contradictory findings was that periodontal bacterial species in a complex-based classification were detected, but periodontitis was not observed[239].

Recently, high-throughput DNA sequencing technology using next-generation sequencing (NGS) methods has been used to comprehensively analyze bacterial composition in different environments[249]. In addition, bioinformatic techniques and pipelines have been improved to analyze data obtained from NGS[250]. In particular, NGS technology is suitable for bacterial analysis because the 16S rRNA genes from bacteria critically define all bacterial species and have an approximate length of 1 kbp of nucleotide sequences that are highly conserved among bacterial species[251]. Therefore, NGS technology combined with bioinformatic techniques is a useful tool for scientists as it allows for a comprehensive evaluation of microbial composition within the microbiome. Griffen *et al* compared the bacterial composition of subgingival plaque between patients with chronic periodontitis and periodontally healthy controls using 16S pyrosequencing. They reported that the proportion of red complex microbes was high at the periodontitis sites, but the microbiome included bacterial species that were not previously regarded as being involved in the etiology of periodontitis[252]. This result showed that conventional methods, such as culture methods, real-time polymerase chain reaction, or checkerboard DNA-DNA hybridization, were limited by the number of bacteria that could be detected. However, current methods using NGS are not limited to the number of bacterial species that can be detected. A few studies using NGS with the 16S rRNA gene region suggested that several taxa, including *Filifactor alocis* and TM7, showed greater abundance in periodontitis and seemed to act as the core bacteria in the

subgingival microbiome of disease in addition to the bacteria thought to be conventional periodontal pathogens[252, 253]. In other words, these results suggest that bacteria other than conventional periodontal pathogens, such as red complex members, also contribute to periodontitis, and dysbiosis within the microbiome potentially induces periodontal inflammation.

Hajishengallis *et al*. proposed the "keystone-pathogen hypothesis," wherein a specific bacterial species is considered a keystone periodontal pathogen, such as *P. gingivalis,* which changes the microbial balance and induces inflammation through dysbiosis, despite the low abundance of the pathogen[20],[254]. Several problems with DNA-based analyses alone have indicated that the 16S rDNA profile may include dead bacteria[255]. On the other hand, only viable bacteria can be analyzed by conducting experiments using 16S rRNA[256]. However, analysis of only 16S rRNA is not a reliable index for bacterial activity and may be misleading in some cases because the relationship between rRNA and growth rate differs significantly in subspecies, even if these bacteria belong to the same bacteria at the species level[257]. Therefore, the 16S rRNA/16S rDNA ratio has been useful as a bacterial activity index for detecting highly active bacterial species, which, despite the low abundance of the pathogen, can induce dysbiosis in certain environments[258, 259]. Kachi *et al* clarified that the activity of *P. gingivalis* was lower than that of other species in periodontitis sites, although *P. gingivalis* was abundant in both DNA and RNA. In contrast, *T. forsythia*, *Fusobacterium nucleatum* subsp. *Vincentii, Streptococcus oralis, T. denticola, F. alocis, and Streptococcus salivarius* showed high activity in periodontitis sites[258]. By analyzing both DNA and RNA using the 16S rRNA region, it may be easier to detect keystone pathogens because this analysis can lead to the understanding of not only the abundance of each bacterium but also their activities. However, these analyses alone cannot reveal the functional composition of the microbiome. Tools such as piphillin or PICRUSt can predict the functional composition of the microbiome using the information of the 16 S rRNA region[260, 261]. Ikeda *et al* reported that pathways for phenylpropanoid and GPI-anchor biosynthesis and metabolism of alanine, arginine, aspartate, butanoate, cyanoamino acid, fatty acid,

glutamate, methane, proline, and vitamin B6 were significantly over-represented in periodontitis subjects compared with healthy subjects using piphillin[262]. Thus, we might understand the essence of polymicrobial disease by determining not only the composition of the microbiome but also the functional gene composition.

In terms of bacterial composition analysis, the 16S rRNA region is highly conserved among prokaryotes, such as bacteria and archaea, and the length of the 16S rRNA region is suitable to provide sufficient information for phylogenetic analysis[251]. However, the length of the 16S rRNA region is almost 1600 base pairs. Among bacteria, *Mycoplasma*, which is the smallest parasitic bacterium, has 580,000 base pairs[263]. This also indicates that the 16S rRNA region alone is insufficient for a complete understanding of the microbiome. In addition, 16S rDNA and 16S rRNA sequencing has caused PCR bias[264] and the different results depend on primer type[265]. To compensate for these shortcomings, metagenomic and metatranscriptomic analyses, which decode all DNA and RNA information, have attracted attention. Both metagenomic and metatranscriptomic analyses can analyze bacterial and functional composition, but metagenomic analyses focus more on species-level profiling, metatranscriptomic analyses concentrate more on gene expression levels[266]. A report by Dabdoub *et al*, using metagenomic analysis, reported the microbial and functional differences between healthy and periodontal sites. *Porphyromonas, Fusobacterium, Fretibacterium, Filifactor, Parvimonas, Selenomonas, Treponema,* and *Kingella* were more abundant in periodontitis sites than in healthy sites. In addition, most functional genes in healthy sites were dedicated to energy utilization through oxidative pathways, while fermentation and methanogenesis were the predominant energy transfer mechanisms in the disease site[267]. A study using metatranscriptomic analysis reported that *T. forsythia* and *P. gingivalis* upregulated different TonB-dependent receptors, peptidases, proteases, aerotolerance genes, iron transport genes, hemolysins, and CRISPR-associated genes in periodontitis sites. Interestingly, bacteria that have not been usually implicated in periodontitis, such as *S. oralis*, *S. mutans*, *S. intermedius*, *Streptococcus mitis*, *Veillonella parvula*, and *Pseudomonas fluorescens*, were highly active in transcribing putative virulence factors in periodontitis

sites[268]. Another report using metatranscriptomic analysis revealed that bacteria, such as *Bacteroides massiliensis and Leptotrichia hofstadii* had high transcriptional activity in addition to that of the red complex[8]. Furthermore, combining metagenomic and metatranscriptomic analyses help understand the activity or enrichment of a given gene set in the microbiome[266]. The combination of metagenomic and metatranscriptomic analyses by Duran-Pinedo *et al* showed that *P. gingivalis, T. forsythia, and T. denticola* exhibited a higher mean abundance and gene expression in periodontitis sites than in healthy sites. However, *Bacteroidetes* oral taxon 274, *Corynebacterium matruchotii*, and *L. hofstadii* in periodontitis sites showed a decrease in relative abundance but represented a high proportion of gene expression, when comparing healthy and periodontitis sites. In addition, the vast majority of virulence factors upregulated in periodontitis sites originate from bacteria that are not considered major periodontal pathogens, such as *Neisseria flavescens*, *C. matruchotii, Rothia dentocariosa*[269]. Komatsu *et al* combined metagenomic and metatranscriptomic analyses with a network analysis for periodontitis. Their results showed that *Fusobacterium nucleatum* subsp. *vincentii* had the highest activity in periodontitis sites, followed by *Peptostreptococcus stomatis* and *Leptotrichia* sp. according to the RNA/DNA ratio which are similar to the 16S rRNA/16S rDNA ratio. Additionally, *Fretibacterium fastidious*, and *F. alocis, Eubacterium nodatum* had high activity and were core bacteria in a co-occurrence network of periodontitis sites. In addition, the virulence factors, such as translation initiation factor IF-1 and pillin subunit in metatranscriptomic data were more enriched than in metagenomic data, indicating these virulence factors have high activity in periodontitis sites[270]. These results suggest that there are bacteria that are more active than the red complex in periodontitis sites, those bacteria are involved in the pathogenesis of periodontal disease, and bacterial activity and interaction with each other are changed in response to environmental changes.

Periodontitis is not an infectious disease caused by a single bacterium, such as diphtheria, tetanus, typhoid fever, and leprosy, but a dysbiotic disease caused by alterations in the abundance of keystone and/or accessory pathogens within the polymicrobial

community, leading to inflammatory responses (**Figure 1**). In addition, dysbiotic microbiota further develops and stimulate inflammatory responses[6]. However, it is difficult to comprehensively understand the etiology of periodontitis, although exhaustive DNA and RNA analyses have been performed. There are several possible explanations for this reason. First, many microorganisms, including bacteria, unculturable bacteria, fungi, and viruses, are present in the oral cavity[271]. Second, the same bacteria can act as homeostatic commensals in one environment and accessory pathogens in another. Furthermore, some bacteria can act as keystone or accessory pathogens depending on their condition[6]. Recently, trans-omics has been attracting attention and has focused on connecting multi-omics data, including not only the genome and transcriptome, but also the proteome and metabolome[272]. Trans-omics has been shown to be effective in elucidating various complex mechanisms, such as insulin sensitivity in liver cells and muscle adaptation[273, 274]. These techniques might be applied to unravel the full picture of periodontal disease as a polymicrobial disease.

6. CHARACTERISTICS OF GUT MICROBIOME IN PATIENTS WITH PERIODONTAL DISEASE

Periodontal disease is characterized by a complex relationship between the host inflammation and polymicrobial dysbiosis of the oral-dental biofilm. This leads to chronic inflammation of the periodontal tissue, ultimately leading to bone destruction and tooth loss. However, over the last several years, this dysbiosis of the oral microbiome has been also associated with multiple systemic inflammatory conditions, such as cardiovascular disease, obesity, metabolic syndrome, and rheumatoid arthritis[75, 275, 276]. As mentioned previously, there are two main prevailing hypotheses regarding the potential for localized chronic periodontal infection to cause systemic inflammation. Namely, 1) periodontal dysbiosis and inflammation leads to increase bacterial translocation into the systemic circulation causing inflammatory mediators and immune complexes to be circulated to other organ systems[277, 278], and 2) chronic periodontal dysbiosis leads to

disturbances and changes to the gut microbiome *via* oral ingestion of periodontopathic organisms.

The concept that the oral microbiome drives the gut microbiome space is highlighted by the fact that some of the first bacteria to colonize the gut come from the mouth[279]. Infants born from vaginal deliveries are exposed to the microbiome of the mother's vaginal tract and gastrointestinal tract during labor, while breastfeeding introduces the skin microbiome to the infant[279]. As the gut microbiome matures over time, new bacterial strains are introduced into the gut *via* the environment and foods that we are exposed to in our childhood and young adulthood.

Yet, the established gut microbiome of adults is not stagnant, but rather can be change drastically by the environment, food, and/or medications. The connection of the oral microbiome and periodontal inflammation causing changes in the gut microbiome is highlighted in several animal and human studies. In a study of 44 patients, which included 7 healthy controls, authors found that patients with either gingivitis (n = 14) or chronic periodontitis (n = 23) had lower gut bacterial diversity as compared to healthy controls[21]. Specifically, they saw that patients with chronic periodontitis had higher levels of *Firmicutes, Proteobacteria, Verrucomicrobia* and *Euryarchaeota* and lower levels of Bacteroidetes as compared to controls[21]. In patients with gingivitis, *Prevotella, Comamonadaceae* and *Lactobacillales* were elevated and *Bacteroidales* was decreased as compared to healthy control[21]. Utilizing a random forest classifier, *Mogibacteriaceae, Ruminococcaceae,* and *Prevotella* were able to discriminate individuals with periodontal diseases from healthy controls with an accuracy of 84%. Because of the cross-sectional design of this study, causality could not be inferred. However, several animal models have shown that the oral administration of *P. gingivalis* into mice leads to significant alteration of the gut microbiome, increase in serum endotoxemia, and a reduction of gut barrier function[20, 32]. The oral administration of *P. gingivalis* in mice led to increases in *Bacteroidetes* and a reduction of *Firmicutes*[20, 32]. These results in mice were recently mirrored in a translational study in humans. Bao and Li *et al.* took salivary and fecal samples from 16 healthy participants and 21 patients with severe periodontitis and

transplanted their salivary microbiome into wildtype C57BL6 mice[19]. The authors showed that patients with severe periodontitis had a significantly different gut microbiome than those of healthy controls, with more saliva-sourced microbiome found in the gut of patients with severe periodontitis as compared to controls[19]. When transplanted into mice, the authors showed that by using utilizing carboxyfluorescein diacetate succinimidyl ester stained bacteria the salivary microbiota persist in the gut for at least 24 h[19]. They also showed that the salivary microbiome of patients with severe periodontitis when transplanted into mice lead to an increase in gut levels of *Porphyromonadaceae* and *Fusobacterium*, while at the same time leading to a decreased expression of gut epithelial tight junction proteins and increased levels of pro-inflammatory cytokines. These findings alongside our prior works[11, 280], demonstrate the important role of the oral-gut axis in other systemic diseases.

7. RELATIONSHIP BETWEEN GUT DYSBIOSIS AND NAFLD

Because of the interplay between the gut microbiome and inflammation, many studies have examined the role of the gut microbiome in liver disease. As part of this review, we will only focus on the relationship between the gut microbiome and nonalcoholic fatty liver disease (NAFLD). The term NAFLD encompasses a spectrum of diseases that range from bland steatosis or nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) and advanced hepatic fibrosis or cirrhosis. Several studies have linked gut dysbiosis to each phase of NAFLD.

NAFL. There have been several studies linking the gut microbiome to NAFL with variable results. In adult patients with NAFL, the most common genera that have been elevated were *Lactobacillus* and *Escherichia,* and *Coprococcus* and *Prevotella* were the most common to be reduced as compared to weight matched controls[281-284]. While these studies looked only at composition, several others examined NAFL utilizing a multi'omics approach, which combined sequencing data with metabolomics. Raman *et al* have shown that 18 stool metabolites, including increased levels of acetic acid, butanoic acid, and propanoic acid, are associated with NAFL in adult patients[285]. Da Silva et al.

also found a characteristic enrichment of isobutyric acid and propionic acid in the feces of NAFL patients[283]. However, one of the most convincing data that has shown the causative relationship between the gut microbiome and NAFL comes from the translational study performed by Hoyles *et al*[284]. Utilizing hepatic transcriptome, gut metagenome, and serum and urine metabolome, they saw NAFL was associated with an increased serum level of several branched-chain and aromatic compounds, including phenylacetic acid[284]. In addition, administration of phenylacetic acid into mice transplanted with human fecal microbiota caused hepatic steatosis[284].

NASH. While studies examining the profile of NAFL were slightly mixed based on the population being investigated, studies examining the profile of NASH to obese controls showed more consistent findings[286]. In adults, NASH patients had lower levels of *Faecalibacterium, Ruminococcus and Bifidobacterium*[283, 287] and a higher level of *Lactobacillus*[288]. Few studies have examined the metabolomics of NASH patients separate from NAFL, likely due to the reason that a diagnosis of NASH requires a liver biopsy to confirm. However, in one study of 16 adults diagnosed with NASH by biopsy, NASH patients had an increased ratio of primary to secondary bile acids which correlated with increase hepatic injury and inflammation[289].

NAFLD-related Advanced Fibrosis. Advanced fibrosis is defined as a fibrosis stage of 3 or more and is associated with higher risk for morbidity and mortality. In general, microbiome studies of NAFLD-related advanced fibrosis usually sees a decrease in microbial diversity marked by an increase in gram-negative bacteria[290-293]. Studies have found that NAFLD-related fibrosis was associated with an increase level of *Bacteroides* and *Escherichia*,[290-292] with mixed results with other genera like *Prevotella*[291, 293, 294]. Metabolite studies found that 3-phenylproanoate[293] and 3-(4-hydroxyphenyl) lactate[295] were associated with NAFLD-related advanced fibrosis. 3-(4-hydroxyphenyl) lactate was also strongly correlated with several bacterial species that were also associated with hepatic fibrosis, such as *Escherichia coli, Bacteroides caccae* and *Clostridium sp*[295].

The mechanisms by which the gut microbiome can cause steatosis, inflammation, and hepatic fibrosis is highlighted in animal models. Patients with NAFLD and obesity are associated with a microbiome that leads to decreased gut epithelial barrier function[296]. This disruption in the gut epithelium leads to increased bacterial translocation, higher levels of endotoxemia, and subsequent increased activation of toll-like receptor signaling[297, 298]. However, a clinical trial utilizing toll-like receptor inhibitor did not show any benefit in patients with NASH.[299] Other mechanisms, include alterations of short-chain fatty acids[300] and bile acid metabolism[301] and signaling[302].

8. PERIODONTAL APPROACHES IN THE PREVENTION AND TREATMENT OF NAFLD

8-1. Management of oral microbiota with conventional periodontal treatment

Periodontal treatment is based on the mechanical and chemical removal of dental plaque and pathogenic factors from the root surfaces by the patient or by a specialist to eliminate inflammation in the periodontal tissues and promote wound healing in the host. It is known that patients who achieve good oral hygiene and a healthy morphology of periodontal tissues through periodontal treatment show a decrease in the total number of bacteria and the proportion of periodontal pathogens in saliva and plaque, as well as a change in the composition of the oral microbiota to a healthy state. Therefore, given the relationship between periodontal disease and NAFLD supported by many epidemiological studies, periodontal therapeutic interventions may be a preventive measure for NAFLD (**Figure 2**).

In a cross-sectional study of 6,352 adults participating in the Korean National Health and Nutrition Examination Survey, Kim *et al*[303]reported that there was an inverse association between frequency of tooth brushing and NAFLD prevalence (fatty liver index ≥60), with a modified odds ratio of 0.56 for NAFLD in the group that brushed three or more times a day compared with the group that brushed once a day or less. A few intervention studies have examined the effect of periodontal treatment on NAFLD. Yoneda *et al*[37] reported that in a single-arm intervention study, non-surgical periodontal therapy, including oral

hygiene instruction and scaling/root planing (SRP) to 10 patients with NAFLD who had 4 or more sites with periodontal pockets ≧ 5 mm led to a significant improvement in AST and ALT at 3 mo after treatment. Interestingly, a recent multicenter randomized controlled trial by Kamata *et al*[304] demonstrated the efficacy of periodontal therapy for patients with NAFLD and periodontal disease. The control group (n = 20) received oral hygiene instruction only, while the experimental group (n = 20) received oral hygiene instruction and SRP. Serum levels of ALT and anti-*P. gingivalis* immunoglobulin antibody titers at 12 and 60 weeks after treatment were measured as primary endpoints. Results showed significant reductions in ALT levels, endotoxin levels, and liver fat content in the SRP group compared to the toothpaste group. In addition, the decrease in *P. gingivalis* IgG antibody titer was significantly higher in the SRP group than in the toothpaste group. These decreased ALT levels and *P. gingivalis* IgG-antibody titers in the SRP group were sustained until 60 weeks from baseline. The SRP group showed significantly greater reductions in blood lipid parameters, including total cholesterol, LDL-cholesterol, and triglycerides at 12 weeks post-treatment, while no significant changes in glycometabolism parameters such as glucose and insulin.

Several studies presented in this section support the potential effect of periodontal treatment in improving outcomes in NAFLD. They also strengthen the causal relationship between periodontal disease and NAFLD. However, only one high-quality randomized controlled trial has been reported to date to assess treatment efficacy, and the relationship between the two is not clear. In the future, more evidence will need to be accumulated, and these controlled trials will need to be systematically meta-analyzed.

8-2. Oral and gut microbiome-targeted therapy using probiotics, prebiotics and bacteriocins

If opportunistic pathogens and periodontopathic bacteria that predominate in oral microbiome changes cause subsequent gut microbiome dysbiosis, an approach of microbiome-targeted therapy with antibiotics, probiotics and prebiotics may help prevent the onset and progression of NAFLD for patients with periodontal disease[11,

[24] (**Figure 2**). Probiotics are defined as live cultured microorganisms that provide health benefits in humans and animals when consumed, generally by improving or restoring the gut microbiota. While, prebiotics are defined as compounds in food that induce the growth or activity of beneficial microorganisms such as bacteria and fungi by altering the composition of organisms in the gut microbiome. When probiotics and prebiotics are used in combination, this is referred to as symbiotics. On the other hand, antibiotics exert beneficial effects on metabolic disorders by non-specifically suppressing the microbiome but may be accompanied by harmful side effects and potential emergence of antibiotic-resistant bacterial strains. Thus, currently, supplementation with probiotics and prebiotics in the treatment of NAFLD and periodontal disease has been favorably accepted due to a potential improved safety for humans and the environment[305-307].

As for treatment of NAFLD, multi-strain probiotic VSL#3 (contains eight species of *Streptococcus thermophilus*, *Bifidobacteria* [*B. breve*, *B. infantis*, *B. longum*], *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, *L. bulgaricus*) was originally developed to treat inflammatory bowel disease, but VSL#3 administration has also been reported to improve fatty liver and fibrosis for NAFLD[308-310]. A randomized controlled trial by Eslamparast *et al* in patients with NAFLD showed that compared to the placebo group, a symbiotic consisting of a probiotic cocktail similar to VSL#3 and fructo-oligosaccharides given twice daily for 28 wk significantly improved liver function, inflammation, and fibrosis grade[311]. Furthermore, a systematic meta-analysis of 21 randomized clinical trial concluded that the consumption of probiotics or symbiotics in NAFLD patients is an effective treatment modality to improve liver function, liver hardness, and blood markers of liver damage[305].

The effect of probiotics on periodontal treatment have also been reported to have several benefits in animal and clinical studies[312]. In studies using probiotics as monotherapy or as adjunctive therapy, *Lactobacillus salivarius* WB21, *L. plantarum*, *L. reuteri*, *L. rhamnosus* SP1, *Bacillus lactis*, and various *Streptococci* showed some efficacy in suppressing periodontopathic bacteria or other anaerobic bacteria. In particular, some *Lactobacillus* spp. have been noted to play a protective role against both NAFLD and periodontal

disease[313-315]. Despite limited, beneficial effects were also observed in improving clinical parameters of periodontal disease, such as plaque score, gingival inflammation, and host immune response factors.

In recent years, our research team has reported multiple times that the use of nisin, an antimicrobial peptide produced by *Lactococcus lactis*, is effective in treating periodontal disease[312, 316-319]. Nisin is a type of bacteriocin that is widely used in the food industry as well as in the medical field because of its potent and broad-spectrum antimicrobial activity even in small amounts, despite its low cytotoxicity and low incidence of bacterial resistance[320-322]. Nisin is classified as a Class I bacteriocin, and is a lantibiotic (lanthionine-containing antibiotics) based on its chemical structure, because it has abnormal amino acids that are caused by translational modifications[323-325]. With regard to the mechanism of action as an antimicrobial substance, nisin attaches to lipid II present in the bacterial cell membrane, and eight molecules of nisin form a tunnel-like pore with four molecules of lipid II, thereby promoting the outflow of intracellular ions, ATP and other substances from the bacteria[326-329]. The production of bacteriocins, such as nisin, is strictly regulated by bacterial signaling *via* quorum sensing, which works together with the self-tolerance capacity of bacteriocin-producing bacteria to selectively control the bacterial flora[330].

Interestingly, we found that in artificial oral biofilms derived from saliva, administration of the nisin-producing probiotic *L. lactis* or nisin significantly inhibited the biofilm formation and bacterial viability, reducing periodontal pathogens while maintaining endogenous oral bacteria such as *Neisseria* spp[317]. Similarly, wild type *L. lactis* or nisin dose-dependently reduced the viability of artificial biofilms formed on titanium alloy discs mimicking peri-implantitis and shifted the composition, relative abundance, and diversity level of the biofilms toward a healthy state with improved proportions of *Proteobacteria* and *Firmicutes* phylum[318]. Furthermore, in a polymicrobial mouse model of periodontal disease infected with multiple periodontopathic bacteria, we have shown that oral administration of nisin-producing *L. lactis* or nisin suppressed periodontal inflammation and alveolar bone loss[316]. These microbial treatments

improved oral dysbiosis and decreased serum antibody responses to periodontal pathogens. On the other hand, non nisin-producing *L. lactis* also reduced the oral and systemic impacts of the periodontal polymicrobial infection, but its effects were not as pronounced as those of nisin-producing *L. lactis*. Surprisingly, nisin and the *L. lactis* probiotic also induced a reparative phenotype *in vivo* in this polymicrobial disease model of periodontal disease. These results suggest that the combined interaction of living bacteria and their bacteriocins is important for probiotic use to be effective.

However, the significance of microbiome-targeted therapy in the treatment of periodontal disease-related NAFLD has been studied to a limited degree. In this regard, we are currently exploring the prophylactic effects of nisin on periodontitis-induced gut dysbiosis and liver disease using the polymicrobial infection mouse model[251]. Detailed analysis of NAFLD-related genes and glycolipid metabolism in the liver is still ongoing in our lab, but we found that nisin shifts the oral and gut microbial composition to a healthy state and mitigates inflammatory responses. Nisin may also prevent hepatic lipid deposition by regulating mitochondrial functions and oxidative stress production in liver tissue.

Few studies have evaluated the effects of *L. lactis* probiotics or nisin in NAFLD, but some evidence based on *in vivo* experiments has been reported [331-333]. Lee *et al* (2020) showed that oral administration of *L. lactis* NZ3900 strain pre-stimulated with nisin significantly protected fatty liver formation and progression of early atherosclerosis in a rabbit model fed a high cholesterol diet [332]. In a study by Naudin *et al* (2020) using mice fed a high calorie Western diet, compared to control mice taking the beneficial bacterium *Lactobacillus rhamnosus* GGL, oral administration of *L. lactis* subsp cremoris improved glucose tolerance and reduced serum cholesterol levels, weight gain, and obesity, resulting in less hepatic lipid deposition and inflammation[333]. Jena *et al* also reported that *Lactococcus* plays a protective role against liver inflammation in mice whose intestinal environment has been compromised by a Western diet[331]. Although human studies on this topic are very limited, Ansari *et al* showed that a fermented herbal formula

containing *L. lactis* effectively improved serum liver function markers and liver fat deposition[334].

In summary, the use of probiotics, prebiotics, and bacteriocins may be a potential therapeutic strategy for patients with periodontal disease and NAFLD. These microbiome-targeted therapies have been shown to be safe for human health and easy to use on a daily basis in an environmentally friendly manner, so they may be applied alone or in combination with periodontal therapy. The fact that adjuvant therapy with probiotics in periodontal treatment has already been applied clinically will also support the practical application of this new strategy. However, the development of microbiome-targeted therapies for NAFLD is in its infancy and further elucidation is warranted regarding the effectiveness of microbiome-targeted therapies on host immunomodulatory mechanisms, efficient intestinal delivery methods, effective combinations of various probiotics and bacteriocins, and methods for long-term maintenance of microbial composition and functional changes.

CONCLUSION

A growing body evidence from multiple angles proposes that periodontal disease, accompanied by oral inflammation and pathological changes in the oral microbiome, induces gut dysbiosis and is involved in the pathogenesis of NAFLD [11, 24] (Figure 1). The emerging concept of periodontitis-associated oral dysbiosis strongly implicates the role of the oral-gut-liver axis in the pathogenesis of NAFLD, based on the close relationship between the gut and liver connected by the enterohepatic circulation. This is due to the advancement of innovative technologies that enable comprehensive exploration of the microbiota through the widespread use of next-generation sequencing, which has fundamentally changed the definition of periodontopathic bacteria that has persisted in periodontology, and now highlights a turning point in rethinking the relationship between periodontal disease and systemic disease.

The liver is not merely a passive organ; NAFLD itself enhances insulin resistance and increases the risk of diabetes, atherosclerosis, and myocardial infarction with

exacerbation of metabolic syndrome[335-337]. This means that even if NAFLD does not lead to end-stage liver disease, such as cirrhosis or cancer in individuals with metabolic syndrome, it can promote extrahepatic complications that can lead to significant mortality-related disease. Thus, both periodontal disease and NAFLD are interrelated with various elements of metabolic syndrome, leading to a vicious cycle of systemic inflammation and metabolic disturbance through amplification of these disease triangles.[338] Furthermore, in recent years, the liver from patients with NAFLD, as in the elderly and post-cardiac arrest donors, is a marginal organ that increases the risk of poor prognosis after liver resection or liver transplantation, the main treatment modalities for liver disease[339, 340]. The detailed pathomechanisms of hepatic ischemia-reperfusion injury and post-transplant rejection in these operations are not known, but periodontal disease may be a potential risk factor for these hepatotoxic events as well[339, 341-343].

Thus, oral and gut microbiome-targeted therapy based on management of periodontal infections would hold great promise for preventing the onset and progression of NAFLD and subsequent complications (**Figure 2**). Studies suggesting the efficacy of conventional periodontal treatment and microbiome-targeted therapy in patients with periodontal disease and NAFLD are increasing each year, but these are still in their infancy. Most reports of the relationship between periodontal disease and gut microbiome are based on data from animal studies, and a few studies have been conducted in humans. However, given the high prevalence of periodontal disease in recent years and the global increase in NAFLD, this new approach appears to be of great clinical and public health importance. In the future, long-term, large sample size cohort studies and randomized controlled trials should be conducted on this topic, in addition to further accumulation of rigorously controlled basic research.

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