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ORIGINAL ARTICLE

Microbiome composition comparison in oral and atherosclerotic plaque from patients with and without periodontitis

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Abstract

There is no conclusive evidence regarding a causal relationship between periodontitis and atherosclerosis. In this study, we examined the microbiome in the oral cavity and atheromatous plaques from atherosclerosis patients with or without periodontitis to investigate the role of oral bacteria in the formation of atheromatous plaques. We chose four patients with and without periodontitis, who had undergone carotid endarterectomy. Bacterial samples were extracted from the tongue surface, from periodontal pocket (during the oral examination), and from the atheromatous plaques (APs). We investigated the general and oral conditions from each patient and performed next-generation sequencing (NGS) analysis for all bacterial samples. There were no significant differences between both groups concerning general conditions. However, the microbiome patterns of the gingival pocket showed differences depending on the absence or presence of periodontitis, while those of the tongue surface were relatively similar. The microbiome pattern of the atheromatous plaques was entirely different from that on the tongue surface and gingival pocket, and oral bacteria were seldom detected. However, the microbiome pattern in atheromatous plaques was different in the presence or absence of periodontitis. These results suggested that oral bacteria did not affect the formation of atheromatous plaques directly.

Keywords Microbiome · Atherosclerosis · Periodontitis · Next-generating sequencing · Oral bacteria

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Abbreviations

NGS	Next-generation sequencing
LDL	Low-density lipoprotein
Pg	*Porphyromonas gingivalis*
Aa	*Aggregatibacter actinomycetemcomitans*
Co	*Capnocytophaga ochracea*
rRNA	Ribosomal RNA
PPD	Pocket probing depth
BOP	Rate of bleeding on probing
PCA	Principal component analysis

Introduction

Periodontitis is a predominant oral infectious disease in which an excessive immune response directed at the microbiome on the tooth surface destroys the periodontal tissue, forming periodontal pockets. The microbiome in these pockets includes pathogenic anaerobic bacteria that can form biofilms, which are inherently more resistant to antibacterial compounds and host immune components [1]. The mature

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biofilm causes further periodontitis progression because of the prolonged inflammation associated with the protracted immune response. Therefore, periodontitis has two main features: it is an infectious disease caused by microbiome imbalance and a chronic inflammatory disease caused by a dysregulated immune response.

These two characteristics of periodontitis are shared by various systematic diseases, including diabetes, arteriosclerosis, cardiovascular diseases such as stroke and infective endocarditis, brain diseases, cancer, and non-alcoholic steatohepatitis, and also these features increase the risks of preterm birth and low birth weight [2–9]. Arteriosclerosis includes atherosclerosis, in which an atherosclerotic plaque is formed on the blood vessel walls [10]. Briefly, the mechanism of plaque formation involves an endothelial dysfunction with various causes, such as hypertension, diabetes, obesity, and high cholesterol levels. All feature the accumulation of low-density lipoprotein (LDL) cholesterol between the intimal and medial endothelial surfaces. Macrophages in atherosclerotic lesions actively participate in lipoprotein ingestion and accumulation giving rise to foam cells filled with lipid droplets, and thus, the accumulation of those foam cells contributes to lipid storage and the consequent atherosclerotic plaque growth [11]. An association between atherosclerosis and periodontitis has been suggested in some epidemiology reports. Moreover, bacterial investigations aimed at detecting periodontal bacteria in the atherosclerotic plaque have been conducted [12–14]. Although observational data support an association between periodontitis and atherosclerotic vascular disease, the data do not yet justify a causative relationship [15]. Multiple common factors that include diabetes, high blood pressure, dyslipidemia, and smoking habits affect the disease progression. However, there are little data concerning the direct involvement of periodontal bacteria in the development of atherosclerotic vascular disease [16, 17].

Porphyromonas gingivalis (*Pg*) is the most prominent pathogen in periodontal disease. Many studied have addressed the association between *Pg* and atherosclerotic vascular disease in clinical and animal models. Some clinical analyses sought to detect the DNA of *Pg* in atherosclerotic plaques [18–21]. Zaremba et al. reported that *Pg* was detected in atherosclerotic plaque of 10 individuals out of 20 by DNA hybridization method [22]. Szulc et al. reported that *Pg* was detected in only 3 samples from patients with coronary artery disease (9.4%), whereas DNA of *Pg* was detected in 15 atheromatous plaques from patients scheduled for carotid endarterectomy (48.4%) [23]. Sliva Filho et al. reported that there was a significant difference in microbial diversity between subgingival biofilm and atheroma plaques; however, 17 identical phylotypes include *Pg* were found in both samples [24]. We also reported a significant correlation between plasma IgG titer levels against *Pg* with LDL cholesterol levels, both of which are high in patients with severe periodontitis [25]. Furthermore, in a mice model, an association between periodontitis and atherosclerotic vascular disease was demonstrated using *Pg* [26]. Ford et al. [27] also reported that intraperitoneal immunizations with *Pg* enhance the levels of serum antibodies to *Pg* and atherosclerosis. Hypercholesterolemia and subsequent arteriosclerosis occur in apolipoprotein E knock-out mice ($ApoE^{-/-}$), which have been used to investigate the association between periodontitis and atherosclerosis. Lalla et al. [2] reported that oral infection with *Pg* accelerates the early atherosclerosis in $ApoE^{-/-}$ mice. Maekawa et al. [28] reported that chronic oral infection with *Pg* accelerates atheroma formation in $ApoE^{shl}$ mice. However, the authors did not detect *Pg* DNA (16S-ribosomal RNA) by PCR in the aortic valve. Therefore, how *Pg* induce atheroma formation is still unknown.

Microbiome analysis has typically involved bacterial culture [29]. However, recent comprehensive bacterial analysis using the gene for 16S-ribosomal RNA (rRNA) has been successful in detecting bacteria that are difficult to cultivate [30]. NGS has become a popular means of examining a large number and volume of samples [31]. In this study, the association between periodontitis and atherosclerosis in the context of the microbiome in the oral cavity and atherosclerotic plaque was investigated by NGS.

Materials and methods

Ethics statement

This study was approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and Okayama University Hospital (Authorization Number: 1603–059) and Brain Attack Center Ota Memorial Hospital (Authorization Number: 121). All enrolled patients provided written informed consent for the use of their resected tissue and oral samples.

Participants

The study focused on 12 patients who visited Brain Attack Center Ota Memorial Hospital between April 2016 and March 2018, and who were diagnosed with internal carotid artery stenosis. The patients were ≥ 40 years of age, underwent carotid endarterectomy, had more than ten teeth, and consented to participate.

Samples

We harvested atheromatous plaques from the carotid artery walls extracted during carotid endarterectomy. Bacteria in the gingival pocket were collected using absorbent

paper points (United Dental Manufactures Inc., Johnson City, TN, USA). Three paper points were inserted into patients' gingival pockets and were harvested after 1 min. We chose the deepest pocket using the six-point examination. Each item was put them into an Eppendorf tube containing PBS. Later, bacteria from the tongue surface were collected using forensic swabs (Sarstedt AG & Co. Nümbrecht, Germany) by wiping the dorsum portion of each tongue several times. Each swab was placed in a tube and immediately stored at − 80 °C until DNA purification. Blood samples were collected from each patient and serum was prepared as previously described [32].

Oral examination

Periodontal examinations were performed to evaluate the average pocket probing depth (PPD) and rate of bleeding on probing (BOP) for each tooth of each patient. The patients were then divided into three groups according to the latest classification system for periodontal diseases and conditions [33]. In particular, we defined four patients into a control group (H1–H4, they belong to the classification as clinical gingival health on an intact periodontium and clinical gingival health on a reduction periodontium/stable periodontitis patient), four patients into a mild periodontitis group (they belong to the classification as periodontitis/stage II: moderate periodontitis; excluded from further analysis), and four severe periodontitis (P1–P4, they belong to the classification as periodontitis/stage III: severe periodontitis with potential for additional teeth loss).

DNA purification

APs were extensively minced using a scalpel and suspended in phosphate-buffered saline (PBS). The collected material from paper points and swabs were resuspended using PBS. One milliliter of each resuspended bacterial sample was transferred to 2 ml Lysing Matrix B tubes (MP Biomedicals, Santa Ana, CA, USA) containing 0.1 mm silica beads and 500 µl ATL buffer (Qiagen, Hilden, Germany). The contents of each tube were homogenized using FastPrep 24 (MP Biomedicals, Santa Ana, CA, USA) for 45 s at 6.5 m/s. Bacterial DNA was extracted using the QIAamp DNA Microbiome Kit (Qiagen, Hilden, German) according to the manufacturer's instructions. The quality and quantity of the DNA were verified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the PicoGreen dsDNA assay kit (Life Technologies, Grand Island, NY, USA).

Library preparation, sequencing, and analysis of 16S rRNA

The V3 and V4 regions of the 16S rRNA were amplified using the forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, and the reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. (The most recent analysis of the variable portions V1–V3 should be preferred; however, we had used V3 and V4 at the time this study was performed.) Thermal cycling conditions were 98 °C for 3 min; 25 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. After PCR clean-up step using AMpure XP Beads (Beckman Coulter, Inc., Brea, CA, USA), a second PCR was performed to add sequencing adapters and dual-index barcodes to the amplicon target to distinguish amplicons from each sample using the same reaction conditions with only eight cycles instead. After the PCR clean-up step, the quality and quantity of the amplicon were verified using KAPA library quantification kit (KAPA Biosystems Inc., Wilmington, MA, USA). Aliquots (5 µL) of the diluted amplicon from each library were combined to form pooling libraries. Six pM of pooling libraries with PhiX was sequenced using the MiSeq® system (Illumina Inc., San Diego, CA, USA). The obtained sequence was analyzed using the CLC Microbial Genomics Module of the CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Briefly, we extracted 5911 bacteria having sequences that passed both those being retained and operational taxonomic units (OTUs) selected based on a 97% similarity with a minimum of ten reads representing each OTU [34]. Principal component analysis (PCA) and clustering analysis were performed using R statistical software [35]. We also performed co-occurrence analysis for the 13 highly detected operational taxonomic units from control and periodontitis samples using the Quantitative Insights Into Microbial Ecology approach (QUIIME 1) [36] (we want to clarify that at the time, we did the analysis of this study; QUIIME 1 was valid. Currently—from January 1st 2018—QUIIME 1 has been replaced by QUIIME2).

Plasma IgG antibody titer test against periodontal bacteria

Plasma IgG antibody titer test against periodontal bacteria was determined as described previously [23, 32, 37, 38]. Bacterial antigens used were sonicated preparations of *Aggregatibacter actinomycetemcomitans* (Aa) Y4, Aa ATC29523, Aa SUNY67, *Eichenerra corrodens* (Ec) FDC1073, *Fusobacterium nucleatum* (Fn) ATCC25586, *Prevotella intermedia* (Pi) ATCC25611, *Prevotella nigrescens* (Pn) ATCC33563, *Capnocytophaga ochracea* (Co) S3, *Porphyromonas gingivalis* (Pg) FDC381, Pg SU63,

T*reponema denticola* (Td) ATCC35405, and *Tannerella forsythia* (Tf)ATCC43037. The sera from five healthy participants without periodontitis (24–29 years of age) were pooled and used to calibrate the analyses. Standard titration curves were prepared using serial dilutions of this pooled control serum. The absorbance of each sample after reaction was defined as an ELISA unit (EU), with 100 EUs corresponding to a 1: 3200 dilution of the calibrator sample [32]. According to the formula for clinical use, the mean ± 2 standard deviations of the controls, based on the reported dataset of IgG titers to individual pathogens among five healthy individuals, was defined as the standard value of 1.

Statistical analysis

The statistical analysis was performed using the Mann–Whitney *U* Test. A *P* value of 0.05 was considered significant and was determined using SPSS Ver. 23 (SPSS Inc., Chicago, IL, USA) for all the experimental results.

Results

General conditions of the patients were evaluated based on age, disease history, body mass index, blood pressure, C-reactive protein, cholesterol, and HbA1c (Table 1). There were no significant differences between both groups in terms of age, sex, other disease such as diabetes, and markers of inflammation and cholesterol. We evaluated the periodontal condition for each patient group from oral examination and plasma IgG antibody titer test (Table 2). Serum IgG antibody titer was significantly higher in those with periodontitis that in control group for *Aggregatibacter actinomycetemcomitans* (*Aa*) Y4, *Aa* ATCC29523, *Aa* SUNY67, *Capnocytophaga ochracea* (*Co*) S3, and *Pg* FDC381.

The microbiome pattern of tongue surface was relatively similar between control samples and periodontitis samples (Fig. 1a). Among them, the ratio of *Filifactor* was significantly higher in periodontitis patients than in the respective controls (Fig. 1b).

The microbiome pattern of gingival pockets was notably different between the control and periodontitis samples (Fig. 2a). The ratio of *Rothia* and *Neisseria* was lower in periodontitis than in control samples. Conversely, the ratios of *Fusobacterium* and *Filifactor* were higher in periodontitis than in control samples (Fig. 2a, b). The ratio of *Desulfobulbus* was significantly higher in periodontitis samples than in controls (Fig. 2b).

The majority of the bacteria found in the APs microbiome belonged to the soil bacterial families *Burkholderiales*, *Bacillale*, and *Rhizobiales*. Their ratios were similar between periodontitis and control patients (Fig. 3). The ratio of *Sphingomonadales* was higher in periodontitis samples than in control samples.

Table 1 Characteristics of study participants

Characteristic	Control ($n=4$)	Periodontitis ($n=4$)	p
Man, no. (%)	4 (100)	4 (100)	
Age, years	74.8 ± 4.3	75.0 ± 3.2	0.884
Current smoker, no. (%)	0	1 (25)	
Known diabetes, no. (%)	2 (50)	2 (50)	
Known hypertension, no. (%)	2 (50)	2 (50)	
Family history of cardiovascular disease, no. (%)	0	1 (25)	
Body-mass index	25.7 ± 3.2	25.4 ± 1.7	0.885
Blood pressure, mmHg			
Systolic	128.5 ± 24.2	134.5 ± 16.3	0.772
Diastolic	69.3 ± 22.3	72.5 ± 14.2	0.663
CRP, mg/dL	0.32 ± 0.32	0.16 ± 0.17	0.386
Leukocyte count, $\times 10^4$/μL	6535 ± 754	6293 ± 983	0.564
Cholesterol, mg/dL	163.3 ± 31.7	187 ± 40.3	0.309
High-density lipoprotein	31.7 ± 5.1	42.1 ± 2.9	0.248
Low-density lipoprotein	79.8 ± 8.7	118.5 ± 33.9	0.248
Triglycerides, mg/dL	234 ± 224.4	164.5 ± 107.3	0.773
HbA1c (NGSP), %	6.1 ± 0.6	6.8 ± 0.9	0.248

Three gingival pocket bacteria from periodontitis (P2, P3, P4; red) and one control sample (H4; blue) were positioned in the center right side of the PCA graph (separated by a red solid circle), three gingival pocket bacteria from control samples (H1, H2, H3), and one gingival bacteria from periodontitis (P4) were located into the center of the plot (separated by a blue solid circle), respectively (Fig. 4). The two circular locations were sufficiently separated. Tongue surface bacteria from periodontitis samples were located in the center left side of the panel (P1, P2, P3, P4) (separated by a red dotted circle). This position was slightly toward to the right side than that of the control samples (H1, H2, H3, H4) (separated by a blue dotted circle). These two circular locations were comparatively closer. The bacteria in atheromatous plaques were located towards the lower middle region of the plot (separated by a green solid circle), and control and periodontitis atheroma samples could not be clearly distinguished between them. The atheromatous plaques bacteria were located far away from the oral samples (tongue surface and gingival pockets).

Similar to the PCA results, bacteria from oral samples (tongue surface and gingival pocket) and atheromatous plaques were completely different (Fig. 5). However, 75%

Table 2 Periodontal disease infection of study participants

Variable	Controls ($n=4$)	Patients ($n=4$)	p
Total no. of teeth	24.8 ± 2.2	19.8 ± 7.4	0.561
Periodontal pocket depth			
1–3 mm, (%)	97.9 ± 1.8	71.4 ± 4.5	0.021
4–6 mm, (%)	2.0 ± 1.8	27.2 ± 4.3	0.021
Over 7 mm, (%)	0.2 ± 0.3	2.9 ± 2.1	0.026
Sites with gingival bleeding (%)	7.8 ± 8.1	24.0 ± 12.8	0.081
Serume IgG antibody titer test against periodontal bacteria			
A. actinomycetemcomitans Y4	− 0.06 ± 0.49	1.85 ± 1.33	0.021
A. actinomycetemcomitans ATCC29523	0.02 ± 0.38	1.96 ± 0.83	0.021
A. actinomycetemcomitans SUNY67	0.04 ± 0.71	2.35 ± 1.21	0.021
C. ochracea S3	− 2.70 ± 2.53	1.94 ± 3.08	0.043
E. corrodens FDC1073	− 0.43 ± 0.91	0.43 ± 1.68	0.564
F. nucleatum ATCC25586	− 0.11 ± 1.52	2.42 ± 3.82	0.248
P. nigrescens ATCC33563	− 0.95 ± 1.44	− 0.63 ± 0.63	0.248
P. intermedia ATCC25611	− 0.22 ± 0.98	0.50 ± 1.37	0.773
P. gingivalis FDC381	− 0.98 ± 0.31	1.83 ± 1.63	0.021
P. gingivalis SU63	− 0.70 ± 0.46	2.58 ± 4.25	0.083
T. denticola ATCC35405	− 0.03 ± 2.14	0.37 ± 1.94	0.564
T. forsythia ATCC43037	− 0.90 ± 0.25	−0.41 ± 0.80	0.248

of atheromatous plaques bacteria from periodontitis were located at the lower part in the cluster and 75% of atheromatous plaques bacteria from controls were located at the upper part of the periodontitis samples.

We evaluated the correlation of the microbiome in atheromatous plaques between the control group and periodontitis group. In both groups, the major bacteria of the network were *Agrobacterium*, *Delftia*, and *Rhizobium*. However, the network around *Cutibacterium* was different between control and periodontitis samples (Fig. 6).

Discussion

In this research, we performed a pilot study regarding a comprehensive microbiome analysis of the internal carotid artery stenosis in patients affected with and without periodontitis. We harvested bacteria samples from the tongue surface, gingival pockets, and atheromatous plaques from each patient and performed NGS analysis. The microbiome in the oral cavity was higher for the periodontal bacterial pathogens *Fusobacterium* and *Filifactor* in the periodontitis group compared to the control group. In particular, the ratio of *Filifactor* on the tongue surface was significantly higher in the periodontitis group in comparison to the control group. Conversely, the ratio of *Rothia* and *Neisseria* in gingival pockets, which are constituents of the normal bacterial flora in a healthy oral cavity, was lower in the periodontitis group than in the control group [39, 40]. Thus, remarkably, the ratio of normal bacteria in gingival pockets and on the tongue surface decreased, while the ratio of pathogenic bacteria increased.

As pathogenic factors for periodontitis, Red complex species (*Pg*, *T. denticola,* and *T. forsythia*) have been the focus of functional investigations [41]. Although there is no doubt regarding their relationship to periodontitis development, the microbiome does not contain just the pathogenic bacteria, but includes a mixture of various and diverse species of bacteria, to comprise the total population ultimately affecting the development of this disease [42]. It has been suggested that 17 novel bacteria including *Filifactor alosis* probably induce periodontitis, even though these bacteria were not previously thought to be periodontitis-specific pathogenic bacteria [43].

The normal bacterial flora in the oral cavity, which was previously disregarded as insignificant, is actually very crucial for periodontitis development or progression. In general, pathogenic bacteria, such as *Pg*, configure the microbiome with the normal bacteria flora [44]. If the balance of pathogenic and normal bacteria in the microbiome is lost for some reason, the microbiome increases its pathogenicity and induces the disease. Therefore, a comprehensive microbiome analysis is necessary to investigate normal as well as pathogenic bacteria composition.

To investigate the possibility that periodontal bacteria might contribute to atheromatous plaques formation directly on the vascular wall by hematogenous spread, NGS analysis was done using the atheromatous plaque samples. Previous reports established that *Pg* induces the expression of vascular cell adhesion molecule 1 from vascular endothelial cells,

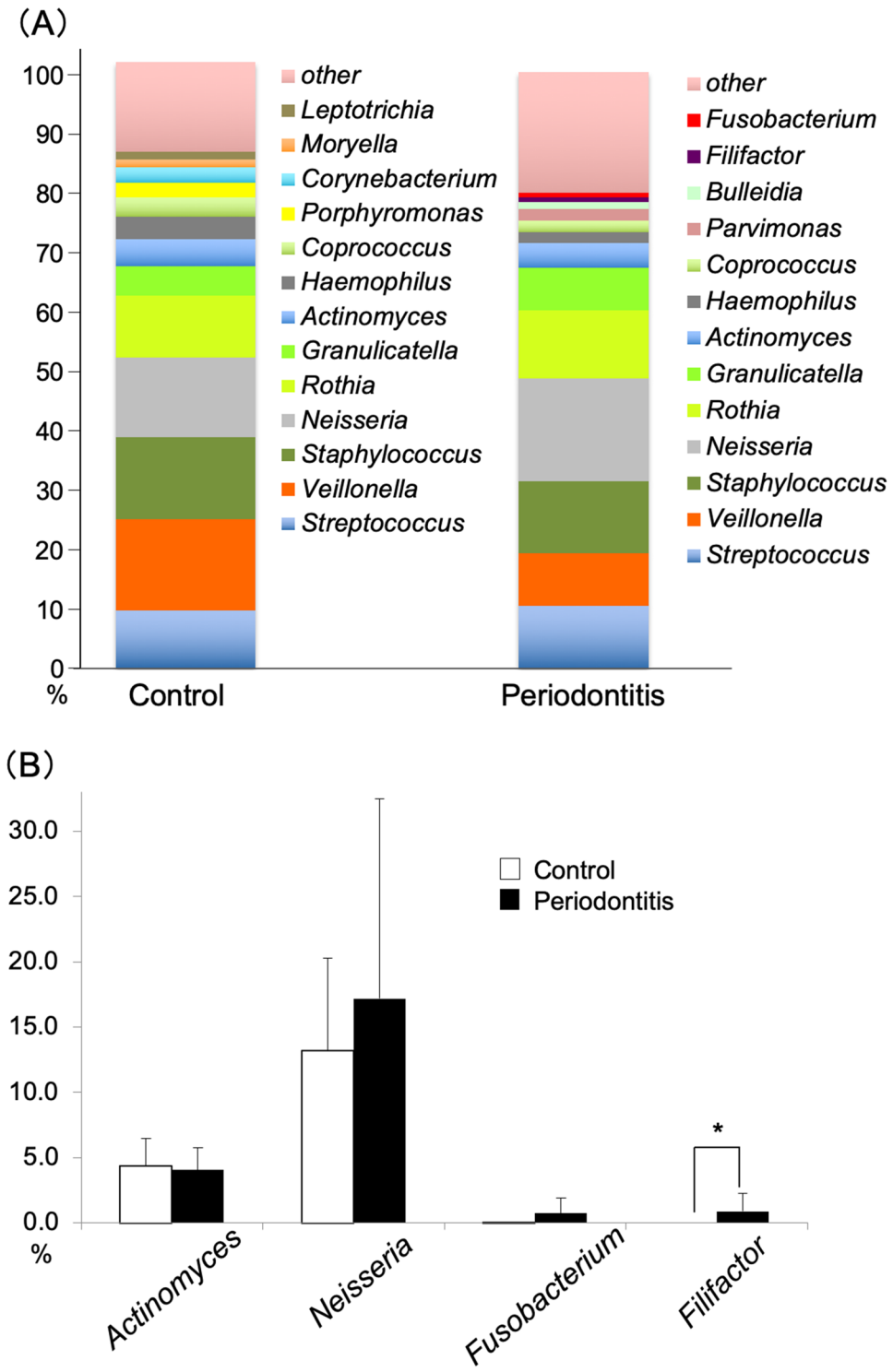

Fig. 1 Characterization of microbiome on the tongue surface. The average ratio of the bacteria on the tongue surface from control and periodontitis patient samples is presented. **a** Bacterial genera are indicated. **b** Thirteen bacterial species were highly detected by NGS analysis. $*P < 0.05$; Mann–Whitney *U* test

and promotes thrombus formation by macrophage invasion into blood vessels, resulting in platelet aggregation [45, 46]. Another report demonstrated that *Pg* infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model [26]. In the present study, oral bacteria were barely detectable in atheromatous plaques, regardless of the presence or absence of periodontitis. The patterns of the microbiome in atheromatous plaques were entirely different on the tongue surface and in gingival pockets. A prior study reported the detection of some oral bacteria in the atheromatous plaques [47]. However, other authors reported that *Pg* infection in an animal model induced atheromatous plaque formation, although this bacterium was actually not detected in the atheromatous plaques [48]. Our

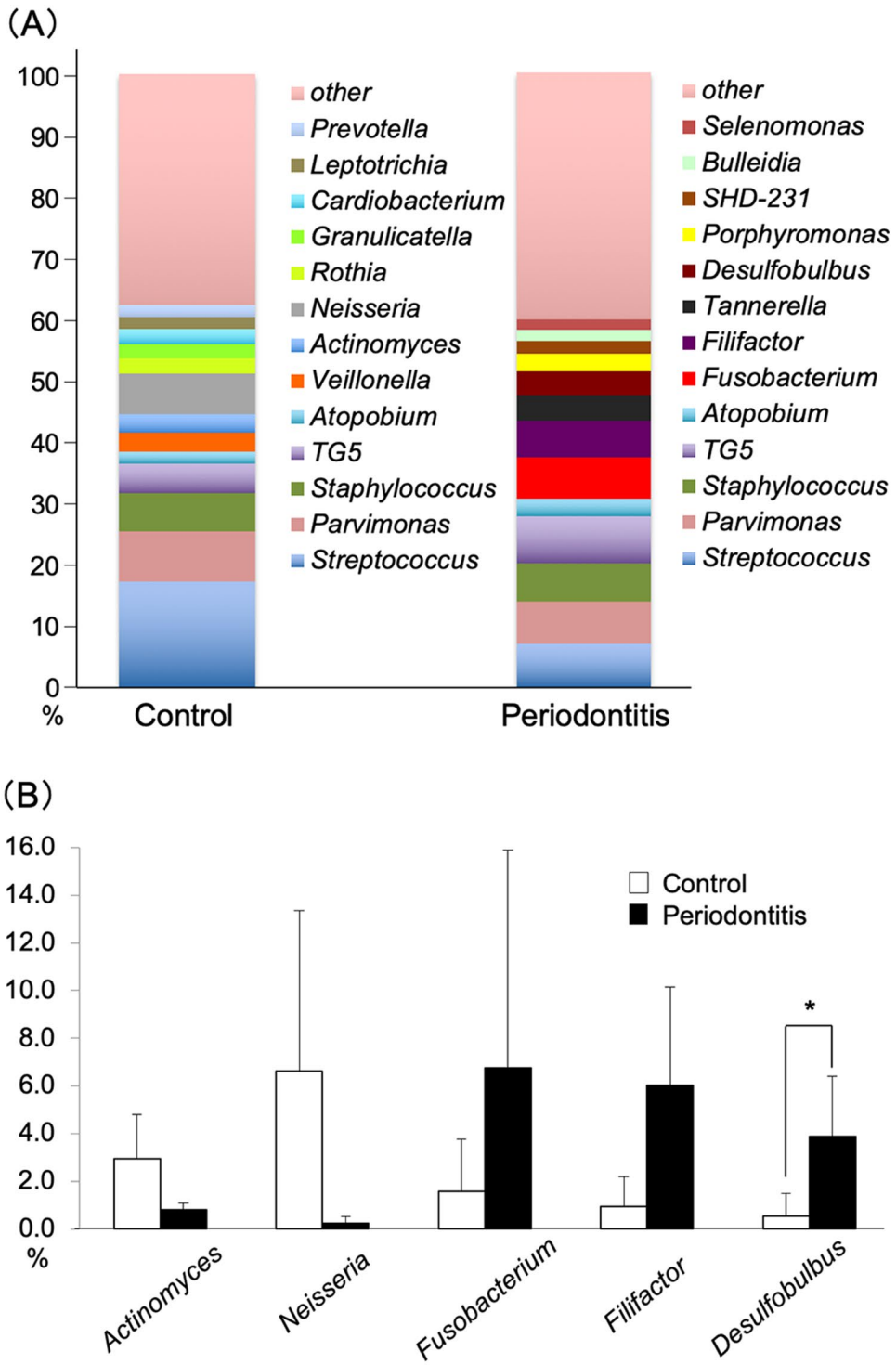

Fig. 2 Characterization of microbiome in gingival pockets. The average ratios of the bacteria in gingival pockets from control and periodontitis samples are shown. **a** Bacterial genera are indicated. **b** Thirteen bacteria that were highly detected from the NGS analysis. $^{*}P < 0.05$; Mann–Whitney *U* test

data enables us to conclude that it is unlikely that the oral bacteria spread hematogenously and directly induce the formation of atheromatous plaque on the aortic wall.

The co-occurrence analysis of the microbiome in atheromatous plaques revealed that the most significant bacteria were *Agrobacterium, Delftia,* and *Rhizobium,* which constituted the network in both groups. Although these are soil bacteria, they were also previously detected in atheromatous plaques [32]. Another significant bacterium, *Cutibacterium,* configured the network in the control group. The relationship with *Cutibacterium* was different among the control and periodontitis groups. This bacterium is categorized as a normal bacterium present on the skin and in the gut, although it was also detected in

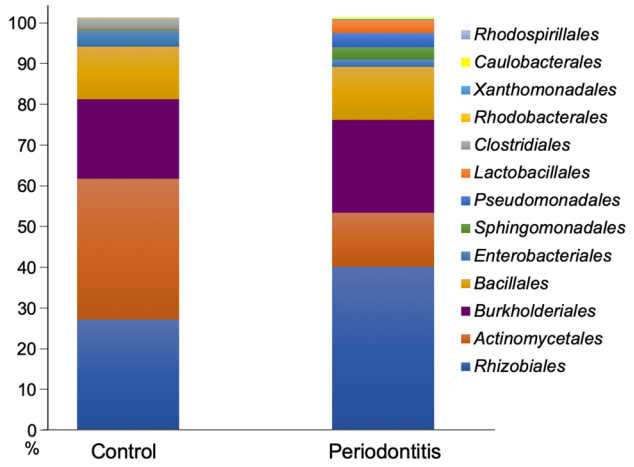

Fig. 3 Characterization of microbiome in atheromatous plaques. The average ratio of the bacteria in gingival pocket from our patients (control and periodontitis samples) is depicted and the order of the bacteria is shown. Thirteen bacteria were highly expressed from the NGS analysis

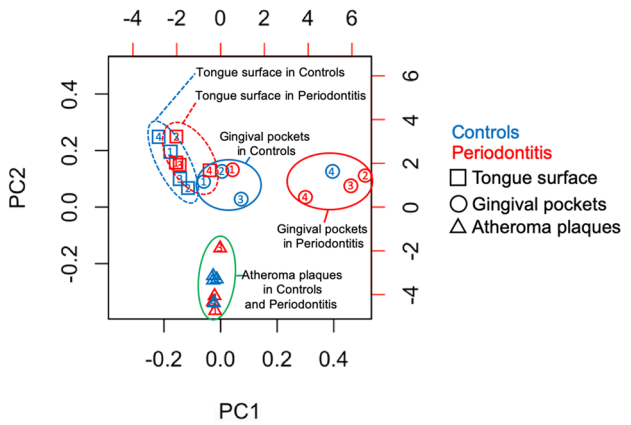

Fig. 4 Comparison of PCA results between control and periodontitis samples. The PCA results from each sample are identified tagged as follows: tongue surface: open square; gingival pocket: open circle; atheromatous plaques: open triangle; controls: blue color; periodontitis: red color. Blue solid circle: three gingival pocket bacteria from control samples and one gingival pocket from periodontitis. Red solid circle: three periodontitis gingival bacteria pocket and one healthy gingival pocket sample. Red dotted circle: four tongue surface from periodontitis samples. Blue dotted circle: four tongue surface from control samples. Green solid circle: four atheroma plaque samples from controls and four atheroma samples from periodontitis

atheromatous plaques [49]. *Cutibacterium* reportedly can cause sarcoidosis, sepsis, and infective endocarditis, and it was also found that heat-killed *Cutibacterium* render mice very susceptible to lipopolysaccharide (LPS) toxicity. *Cutibacterium* also promotes the production of cytokines, such as interleukin-12, interferon-gamma, and Toll-like

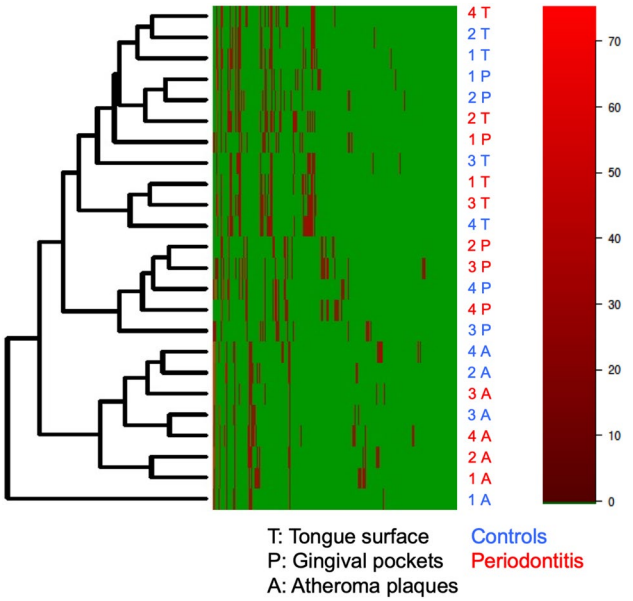

Fig. 5 Comparison of clustering analysis results between control and periodontitis samples. The clustering analysis results from each sample were tagged as follows: T: tongue surface; P: gingival pocket; A: atheromatous plaques. Blue text represents control samples and red text specifies periodontitis samples

receptor 4 [50]. Presently, it is conceivable that LPS produced by periodontal bacteria activated *Cutibacterium* in the blood vessels, which then form the atheromatous plaques. In this scenario, the difference of the network in atheromatous plaques between the control and periodontitis samples might be caused by LPS that is spread hematogenously, as well as by the chronic inflammatory effect. Recently, it was reported that the production of trimethylamine-N-oxide, which promotes atherosclerosis, depends upon the metabolism of the intestinal microbiome [51]. Some previous studies and the present data indicate that the loss of microbiome balance in the human body affects the development of atherosclerosis. Periodontitis has a great effect on the microbiome configuration in the oral cavity and promotes the formation of various metabolic products. The metabolic products of microbiome or the host inflammatory response might indirectly influence the composition of atheromatous plaques. However, this detailed mechanism of atherosclerosis development remains largely unknown. In a further study, we intend to investigate the relationship between periodontitis and atherosclerosis. We also wish to point out the limitations of this study related to the software analysis. These analysis methods are progressing every day. For example, the current segmentation systems have a good behavior in the identification of the genus, although a moderate behavior in the identification of the species was almost null in the strain recognition.

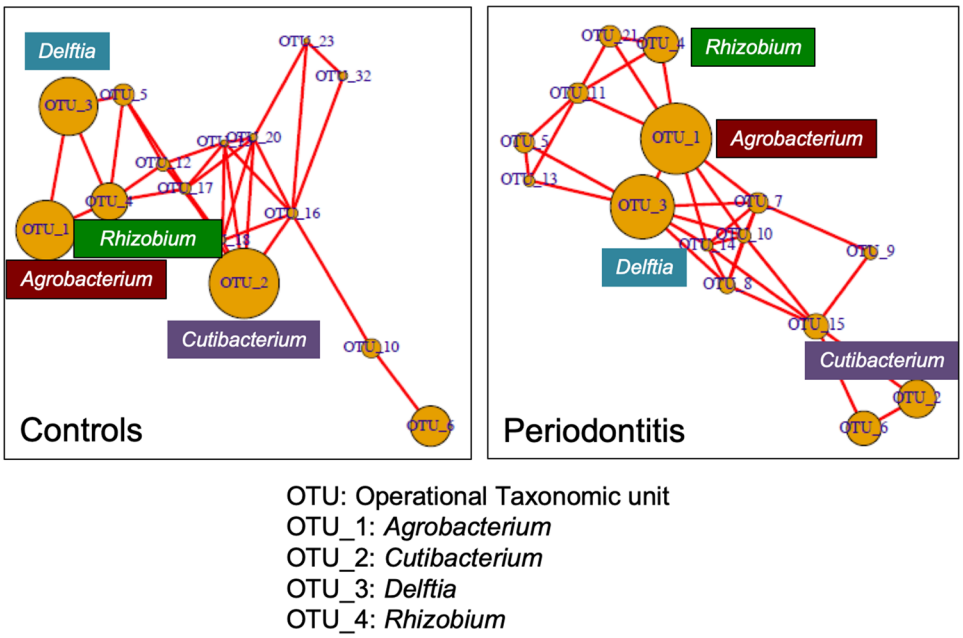

Fig. 6 Co-occurrence analysis of microbiome in atheromatous plaques. Co-occurrence analysis for the 13 highly detected operational taxonomic units (OTUs) from control and periodontitis samples using the Quantitative Insights Into Microbial Ecology approach. Correlation coefficient > 0.4. Abbreviations are: OTU_1: Agrobacterium, OTU_2: Cutibacterium, OTU_3: Delfti, and OTU_4: Rhizobium

Conclusion

The ratio of oral bacteria in atheromatous plaques was remarkably low, and the microbiome pattern in the atheromatous plaques was entirely different from that found in the oral microbiome. There is a possibility that oral bacteria do not directly induce the atheromatous plaque configuration, but this fact was not proved from this study. The microbiome pattern and the correlation of the microbiome in atheromatous plaques were different between the controls and periodontitis samples. Thus, metabolic products of the microbiome, or the host's inflammatory response, might indirectly affect the atheromatous plaque configuration. To confirm this hypothesis, further studies of both epidemiology and animal studies are needed.

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Author contributions DI performed samples collection, conducted the experimental works, interpreted the results, and wrote the draft manuscript. KY supervised all experiments and edited the manuscript. KM performed the oral examination and sample collection. MT performed co-occurrence analysis. SO, MS, and YS operated patients and collected samples. MT and ST performed NGS analysis. ZA performed English editing for the manuscript. TK, KO, and TY gave professional advises. ST was responsible for the study, and supervised and edited the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate This study was approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and Okayama University Hospital (Authorization Number: 1603–059) and Brain Attack Center Ota Memorial Hospital (Authorization Number: 121).

Informed consent We obtained consents for publication from the participants.

References

1. Maddi A, Scannapieco FA. Oral biofilms, oral and periodontal infections, and systemic disease. Am J Dent. 2013;226:249–54 **(PubMed PMID: 24479275)**.
2. Lalla E, Papapanou PN. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. Nat Rev Endocrinol. 2011;7:738–48. https://doi.org/10.1038/nrendo.2011.106.
3. Senba T, Kobayashi Y, Inoue K, Kaneto C, Inoue M, Toyokawa S, et al. The association between self-reported periodontitis and coronary heart disease—from MY Health Up Study. J Occup Health. 2008;50:283–7.
4. Lafon A, Pereira B, Dufour T, Rigouby V, Giroud M, Béjot Y, et al. Periodontal disease and stroke: a meta-analysis of cohort

studies. Eur J Neurol. 2014;21:1155–61, e66-7. https://doi.org/10.1111/ene.12415.
5. Isoshima D, Yamashiro K, Matsunaga K, Shinobe M, Nakanishi N, Nakanishi I, et al. Assessment of pathogenesis of infective endocarditis by plasma IgG antibody titer test against periodontal bacteria. Clin Case Rep. 2017;5:1580–6. https://doi.org/10.1002/ccr3.1066.
6. Dominy SS, Lynch C, Ermini F, et al. Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. Sci Adv. 2019;5:eaaau3333. https://doi.org/10.1126/sciadv.aau3333**(eCollection 2019 Jan)**.
7. Zeng XT, Deng AP, Li C, Xia LY, Niu YM, Leng WD. Periodontal disease and risk of head and neck cancer: a meta-analysis of observational studies. PLoS ONE. 2013;8:e79017. https://doi.org/10.1371/journal.pone.0079017.
8. Yoneda M, Naka S, Nakano K, Wada K, Endo H, Mawatari H, et al. Involvement of a periodontal pathogen, Porphyromonas gingivalis on the pathogenesis of non-alcoholic fatty liver disease. BMC Gastroenterol. 2012;12:16. https://doi.org/10.1186/1471-230X-12-16**(PubMed PMID: 22340817; PubMed Central)**.
9. Sanz M, Kornman K, Working group 3 of the joint EFP/AAP workshop. Periodontitis and adverse pregnancy outcomes: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. J Periodontol. 2013;84:S164–S169169. https://doi.org/10.1902/jop.2013.1340016.
10. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med. 1999;340:115–26. https://doi.org/10.1056/NEJM199901143400207.
11. Vinchi F, Muckenthaler MU, Da Silva MC, Balla G, Balla J, Jeney V. Atherogenesis and iron: from epidemiology to cellular level. Front Pharmacol. 2014;5:94. https://doi.org/10.3389/fphar.2014.00094**(eCollection 2014. Review)**.
12. Bahekar AA, Singh S, Saha S, Molnar J, Arora R. The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: a meta-analysis. Am Heart J. 2007;154:830–7. https://doi.org/10.1016/j.ahj.2007.06.037.
13. Ishihara K, Nabuchi A, Ito R, Miyachi K, Kuramitsu HK, Okuda K. Correlation between detection rates of periodontopathic bacterial DNA in coronary stenotic artery plaque and in dental plaque samples. J Clin Microbiol. 2004;42:1313–5.
14. Okuda K, Ishihara K, Nakagawa T, Hirayama A, Inayama Y. Detection of treponema denticola in atherosclerotic lesions. J Clin Microbiol. 2001;39:1114–7. https://doi.org/10.1128/JCM.39.3.1114-1117.2001.
15. Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME, et al. Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association?: a scientific statement from the American Heart Association. Circulation. 2012;125:2520–44. https://doi.org/10.1161/CIR.0b013e31825719f3.
16. Hujoel PP, Drangsholt M, Spiekerman C, DeRouen TA. Periodontal disease and coronary heart disease risk. JAMA. 2000;284:1406–10.
17. Peacock ME, Carson RE. Frequency of self-reported medical conditions in periodontal patients. J Periodontol. 1999;66:1004–7. https://doi.org/10.1902/jop.1995.66.11.1004.
18. Kurihara N, Inoue Y, Iwai T, Umeda M, Huang Y, Ishikawa I. Detection and localization of periodontopathic bacteria in abdominal aortic aneurysms. Eur J Vasc Endovasc Surg. 2004;28:553–8. https://doi.org/10.1016/j.ejvs.2004.08.010.
19. Beck JD, Garcia RJ, Heiss G, Vokonas PS, Offenbacher S. Periodontal disease and cardiovascular disease. J Periodontol. 1996;67:1123–37.
20. Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. Identification of periodontal pathogens in atheromatous plaques. J Periodontol. 2000;71:1554–600.
21. Nakano K, Inaba H, Nomura R, Nemoto H, Takeuchi H, Yoshioka H, Toda K, Taniguchi K, Amano A, Ooshima T. Distribution of *Porphyromonas gingivalis* fimA genotypes in cardiovascular specimens from Japanese patients. Oral Microbiol Immunol. 2008;23:170–2.
22. Zaremba M, Górska R, Suwalski P, Kowalski J. Evaluation of the incidence of periodontitis-associated bacteria in the atherosclerotic plaque of coronary blood vessels. J Periodontol. 2007;78:322–7.
23. Szulc M, Kustrzycki W, Janczak D, Michalowska D, Baczynska D, Radwan-Oczko M. Presence of periodontopathic bacteria DNA in atheromatous plaques from coronary and carotid arteries. Biomed Res Int. 2015;2015:825397. https://doi.org/10.1155/2015/825397.
24. Serra e silva Filho W, Casarin RC, Nicolela EL Jr, Passos HM, Sallum AW, Gonçalves RB. Microbial diversity similarities in periodontal pockets and atheromatous plaques of cardiovascular disease patients. PLoS ONE. 2014;9:e109761. https://doi.org/10.1371/journal.pone.0109761.
25. Kudo C, Shin WS, Minabe M, et al. Periodontitis and Atherosclerosis Project-Tokyo and Chiba Consortiums. Analysis of the relationship between periodontal disease and atherosclerosis within a local clinical system: a cross-sectional observational pilot study. Odontology. 2015;103:314–21. https://doi.org/10.1007/s10266-014-0172-3.
26. Li L, Messas E, Batista EL, Levine RA, Amar S. Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. Circulation. 2002;105:861–7.
27. Ford PJ, Gemmell E, Timms P, Chan A, Preston FM, Seymour GJ. Anti-*P. gingivalis* response correlates with atherosclerosis. J Dent Res. 2007;86:35–40.
28. Maekawa T, Takahashi N, Tabeta K, Aoki Y, Miyashita H, Miyauchi S, Miyazawa H, Nakajima T, Yamazaki K. Chronic oral infection with *Porphyromonas gingivalis* accelerates atheroma formation by shifting the lipid profile. PLoS ONE. 2011;6:e20240.
29. Papapanou PN, Sellén A, Wennstr JL, Dahlén G. An analysis of the subgingival microflora in randomly selected subjects. Oral Microbiol Immunol. 1993;8:24–9.
30. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014;159:789–99. https://doi.org/10.1016/j.cell.2014.09.053.
31. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. Nature. 2015;517:205–8. https://doi.org/10.1038/nature13828.
32. Kudo C, Naruishi K, Maeda H, Abiko Y, Hino T, Iwata M, et al. Assessment of the plasma/serum IgG test to screen for periodontitis. J Dent Res. 2012;91:1190–5. https://doi.org/10.1177/0022034512461796.
33. Caton JG, Armitage G, Berglundh T, Chapple ILC, Jepsen S, Kornman KS, et al. A new classification scheme for periodontal and peri-implant diseases and conditions—introduction and key changes from the 1999 classification. J Periodontol. 2018;89(Suppl 1):S1–S8. https://doi.org/10.1002/JPER.18-0157.
34. Emery DC, Shoemark DK, Batstone TE, Waterfall CM, Coghill JA, Cerajewska TL, Davies M, West NX, Allen SJ. 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. Front Aging Neurosci. 2017. https://doi.org/10.3389/fnagi.2017.00195**(eCollection 2017)**.
35. Ziganshina EE, Sharifullina DM, Lozhkin AP, Khayrullin RN, Ignatyev IM, Ziganshin AM. Bacterial communities associated with atherosclerotic plaques from Russian individuals with

atherosclerosis. PLoS ONE. 2016;11:e0164836. https://doi.org/10.1371/journal.pone.0164836.
36. Shi W, Qin M, Chen F, Xia B. Supragingival microbial profiles of permanent and deciduous teeth in children with mixed dentition. PLoS ONE. 2016;11:e0146938. https://doi.org/10.1371/journal.pone.0146938.
37. Murayama Y, Nagai A, Okamura K, Kurihara H, Nomura Y, Kokeguchi S, et al. Serum immunoglobulin G antibody to periodontal bacteria. Adv Dent Res. 1988;2:339–45. https://doi.org/10.1177/08959374880020022401.
38. Sugi N, Naruishi K, Kudo C, Hisaeda-Kako A, Kono T, Maeda H, Takashiba S. Prognosis of periodontitis recurrence after intensive periodontal treatment using examination of serum IgG antibody titer against periodontal bacteria. J Clin Lab Anal. 2011;25:25–322.
39. Wang J, Qi J, Zhao H, He S, Zhang Y, Wei S, et al. Metagenomic sequencing reveals microbiota and its functional potential associated with periodontal disease. Sci Rep. 2013;3:1843. https://doi.org/10.1038/srep01843.
40. Takeshita T, Matsuo K, Furuta M, Shibata Y, Fukami K, Shimazaki Y, et al. Distinct composition of the oral indigenous microbiota in South Korean and Japanese adults. Sci Rep. 2014;4:6990. https://doi.org/10.1038/srep06990.
41. Socransky SS, Smith C, Haffajee AD. Subgingival microbial profiles in refractory periodontal disease. J Clin Periodontol. 2002;29:260–8.
42. Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction. Periodontol. 2000;1997(14):9–11.
43. Takeshita T, Nakano Y, Kumagai T, Yasui M, Kamio N, Shibata Y, et al. The ecological proportion of indigenous bacterial populations in saliva is correlated with oral health status. ISME J. 2009;3:65–78. https://doi.org/10.1038/ismej.2008.91.
44. Pérez-Chaparro PJ, Gonçalves C, Figueiredo LC, Faveri M, Lobão E, Tamashiro N, et al. Newly identified pathogens associated with periodontitis: a systematic review. J Dent Res. 2014;93:846–58. https://doi.org/10.1177/0022034514542468.
45. Khlgatian M, Nassar H, Chou HH, Gibson FC, Genco CA. Fimbria-dependent activation of cell adhesion molecule expression in *Porphyromonas gingivalis*-infected endothelial cells. Infect Immun. 2002;70:257–67.
46. Imamura T, Travis J, Potempa J. The biphasic virulence activities of gingipains: activation and inactivation of host proteins. Curr Protein Pept Sci. 2003;4:443–50.
47. Toyofuku T, Inoue Y, Kurihara N, Kudo T, Jibiki M, Sugano N, et al. Differential detection rate of periodontopathic bacteria in atherosclerosis. Surg Today. 2011;41:1395–400. https://doi.org/10.1007/s00595-010-4496-5.
48. Jain A, Batista EL, Serhan C, Stahl GL, Van Dyke TE. Role for periodontitis in the progression of lipid deposition in an animal model. Infect Immun. 2003;71:6012–8 (**PubMed PMID: 14500522; PubMed Central PMCID: PMCPMC201045**).
49. Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci USA. 2011;108:4592–8. https://doi.org/10.1073/pnas.1011383107.
50. Tsutsui H, Imamura M, Fujimoto J, Nakanishi K. The TLR4/TRIF-mediated activation of NLRP3 inflammasome underlies endotoxin-induced liver injury in mice. Gastroenterol Res Pract. 2010;2010:641865. https://doi.org/10.1155/2010/641865.
51. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368:1575–84. https://doi.org/10.1056/NEJMoa1109400.