Human oral, gut, and plaque microbiota in patients with atherosclerosis

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Periodontal disease has been associated with atherosclerosis, suggesting that bacteria from the oral cavity may contribute to the development of atherosclerosis and cardiovascular disease. Furthermore, the gut microbiota may affect obesity, which is associated with atherosclerosis. Using qPCR, we show that bacterial DNA was present in the atherosclerotic plaque and that the amount of DNA correlated with the amount of leukocytes in the atherosclerotic plaque. To investigate the microbial composition of atherosclerotic plaques and test the hypothesis that the oral or gut microbiota may contribute to atherosclerosis in humans, we used 454 pyrosequencing of 16S rRNA genes to survey the bacterial diversity of atherosclerotic plaque, oral, and gut samples of 15 patients with atherosclerosis, and oral and gut samples of healthy controls. We identified *Chryseomonas* in all atherosclerotic plaque samples, and *Veillonella* and *Streptococcus* in the majority. Interestingly, the combined abundances of *Veillonella* and *Streptococcus* in atherosclerotic plaques correlated with their abundance in the oral cavity. Moreover, several additional bacterial phylotypes were common to the atherosclerotic plaque and oral or gut samples within the same individual. Interestingly, several bacterial taxa in the oral cavity and the gut correlated with plasma cholesterol levels. Taken together, our findings suggest that bacteria from the oral cavity, and perhaps even the gut, may correlate with disease markers of atherosclerosis.

The human body is home to microbial ecosystems (microbiotas) whose structure and function differ between different sites in the body (1–6). These microorganisms outnumber the number of eukaryotic cells in the human body by at least an order of magnitude (7). The gut microbiota is the best-studied human-associated ecosystem and has a major impact on our physiology, immune system, and metabolism. For instance, obese individuals generally have a less diverse gut microbiota, and some studies have observed reduced levels of the bacterial phyla Bacteroidetes (3), although others have not (8, 9). Furthermore, germ-free mice have reduced adiposity and are resistant to diet-induced obesity (10, 11). Thus the gut microbiota can be considered an environmental factor that affects obesity. However, the role of the human microbiome in obesity-related metabolic diseases such as atherosclerosis remains to be explored.

Atherosclerotic disease, with manifestations such as myocardial infarction and stroke, is the major cause of severe disease and death among subjects with obesity. The disease is characterized by accumulation of cholesterol and recruitment of macrophages to the arterial wall. It can thus be considered both a metabolic and an inflammatory disease (12). Since the first half of the 19th century, infections have been thought to cause or promote atherosclerosis by augmenting proatherosclerotic changes in vascular cells (13). These changes include increased scavenger receptor expression and activity, enhanced uptake of cholesterol and modified LDL, increased expression of adhesion molecules and inflammatory cytokines, and other effects, such as stimulating macrophages to express cytokines, leading to atherosclerotic plaque vulnerability (13).

Epidemiological studies support an association between cardiovascular disease and infections, such as periodontal disease and *Chlamydia* infections (14, 15). Dental disease has been associated with elevated risk of myocardial infarction (14), and metabolic activity of the gut microbiota was recently shown to relate to blood pressure (16). Furthermore, in a study where bacterial DNA was identified in atherosclerotic plaques, 51.5% of the patients tested positive for *Chlamydia* in their atheromas (17). Several studies suggest an oral source for atherosclerotic plaque-associated bacteria (18–21). However, to date, no single study has directly compared the microbial diversity of oral, gut, and atherosclerotic plaque microbiotas within individuals. This type of cross-site comparison is essential given the high level of variability observed in the microbiota between different subjects (1–6).

Here, we characterized the atherosclerotic plaque, oral, and gut microbiotas obtained from patients with atherosclerosis and healthy controls by pyrosequencing their 16S rRNA genes. Our study addressed the following questions: Is there a core atherosclerotic plaque microbiota? Are bacteria present in the plaque also detectable in the oral cavities or guts of the same individuals? Do the microbiotas of the oral cavity, gut, and atherosclerotic plaque relate to disease markers such as plasma levels of apolipoproteins and cholesterol? Is an altered oral or fecal microbiota associated with atherosclerosis? Our findings revealed a number of phylotypes common to the atherosclerotic plaque and oral and gut samples within individuals, and that the abundances of specific members of the oral and gut microbiota correlated with disease biomarkers.

Results

Overall Comparison of the Human Oral, Gut, and Atherosclerotic Plaque Microbiotas. We surveyed the atherosclerotic plaque, oral cavity (swab from periodontium area), and gut (feces) bacterial communities of 15 patients with clinical atherosclerosis and 15 age- and sex-matched healthy controls (Table 1). The 5′ variable regions (V1–V2) of the bacterial 16S ribosomal RNA (rRNA) gene were PCR amplified using barcoded primers 27F and 338R (22). We generated a dataset of 380,501 high-quality 16S rRNA sequences

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Table 1. Characteristics of study participants

	Controls (n = 15)	Patients (n = 15)	P value
Males, n (%)	12 (80)	12 (80)	NA
Age, years	70.5 ± 0.5	65.7 ± 0.5	0.67
Current smoker, n (%)	0	6 (40)	0.017
Known diabetes, n (%)	0	4 (27)	0.10
Previous myocardial infarction, n (%)	0	4 (27)	0.10
Previous or current cerebrovascular disease			NA
No.	15 (100)	0	
Amaurosis fugax, n (%)		4 (27)	
Transitory ischemic attack, n (%)		6 (40)	
Stroke, n (%)		5 (33)	
Known hypertension, n (%)	2 (13)	11 (73)	0.003
Systolic blood pressure, mm Hg	141 ± 23	146 ± 18	0.35
Diastolic blood pressure, mm Hg	80 ± 12	77 ± 12	0.60
Total cholesterol, mmol/L	5.56 ± 1.12	4.67 ± 1.53	0.026
HDL cholesterol, mmol/L	1.64 ± 0.44	1.27 ± 0.28	0.015
LDL cholesterol, mmol/L	3.35 ± 0.01	2.60 ± 1.36	0.019
Triglycerides, mmol/L (median [interquartile range])	1.21 (0.75)	1.47 (0.80)	0.046
Apolipoprotein AI, g/L	1.43 ± 0.19	1.32 ± 0.21	0.19
Apolipoprotein B, g/L	1.10 ± 0.30	0.98 ± 0.35	0.25
hsCRP, mg/L (median [interquartile range])	0.68 (3.01)	1.12 (3.73)	0.20
Statin treatment, n (%)	0	11 (73)	NA
Antiplatelet treatment, n (%)	0	15 (100)	NA

NA, not applicable due to selection criteria.

(n = 73: 15 patient and control fecal samples, 14 patient oral samples, 15 control oral samples, and 14 atherosclerotic plaque samples). Sequences were assigned to species-level operational taxonomic units (OTUs) using a 97% pairwise-identity cutoff, and chimera checking revealed that 3.1% of total sequences were putative chimeras. One atherosclerotic plaque sample was excluded from the downstream analysis due to low sequence counts (<1,700 sequences). The final dataset included representatives of 13 bacterial phyla; the majority of the sequences were classified as Firmicutes (63.8%), Bacteroidetes (11.7%), Proteobacteria (15.4%), and Actinobacteria (6.4%).

We compared the overall bacterial community composition using the unweighted UniFrac distance metric, a phylogenetic tree-based metric ranging from 0 (distance between identical communities) to 1 (distance between totally different communities with no shared ancestry). This analysis revealed strong clustering of samples by body site, with the atherosclerotic plaque samples forming a distinct cluster apart from oral and fecal samples, indicating all three sites have distinct microbial communities (Fig. 1 and Fig. S1). We used the unweighted (qualitative) rather than the weighted (quantitative) version of the metric because it generally performs better for resolving human body sites (4). The average phylogenetic diversity (23) of the microbiotas was similar for atherosclerotic plaque (AP) and oral cavity (OC, ratio AP/OC= 1.09), but highest for the gut (G; AP/G = 0.7, OC/G = 0.64).

Characterization of the Atherosclerotic Plaque Microbiota. The atherosclerotic plaque contained low but detectable amounts of bacterial DNA: qPCR analysis revealed a positive correlation (ρ = 0.68, P = 0.009) between the amount of bacterial 16S rDNA and the number of leukocytes within the atherosclerotic plaques, suggesting that the amount of bacteria contributes to the inflammatory status of the atherosclerotic plaque. Compared with the oral and gut samples, the atherosclerotic plaque contained significantly higher levels of Proteobacteria and fewer Firmicutes (Fig. 2 *A*–*E*). We detected several OTUs present in all atherosclerotic plaque samples, and that differentiated these samples from oral and fecal samples. For instance, *Chryseomonas* (recently reclassified as *Pseudomonas luteola*), was detected at high levels in the atherosclerotic plaque samples and not at all in the gut or oral samples; furthermore, nearest shrunken centroids (NSC) analysis revealed that this OTU was the most discriminant genus between sites (i.e., driving the differences between the microbiotas of the different body sites; Fig. 3 *A* and *B*). In addition, three OTUs belonging to the genus *Staphylococcus*, three OTUs classified as Propionibacterineae, and one OTU belonging to the genus *Burkholderia* (Fig. 3 *A* and *B*) were specific for atherosclerotic plaques and present in all samples. Together, these observations support the notion that a "core" microbiota composed of the same genus-level lineages exists in atherosclerotic plaque.

Composition of the Oral Microbiota and Its Relation to Atherosclerosis and Disease Markers. The oral microbiota of patients and healthy controls was dominated by Firmicutes (69% and 76% of OTUs classifiable to the phylum level, respectively), followed by Bacteroidetes (10% and 6%), Actinobacteria (9% and 10%), Fuso-

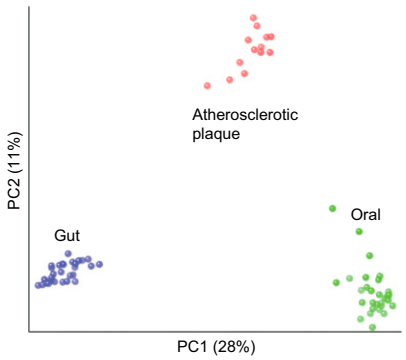

Fig. 1. Bacterial diversity clustering by body habitat. The first two principal coordinates (PC1 and PC2) from the principal coordinate analysis of unweighted UniFrac are plotted for each sample. Each symbol represents a sample, colored by body habitat (green, oral cavity; red, atherosclerotic plaque; purple, gut). The variance explained by the PCs is indicated in parentheses on the axes.

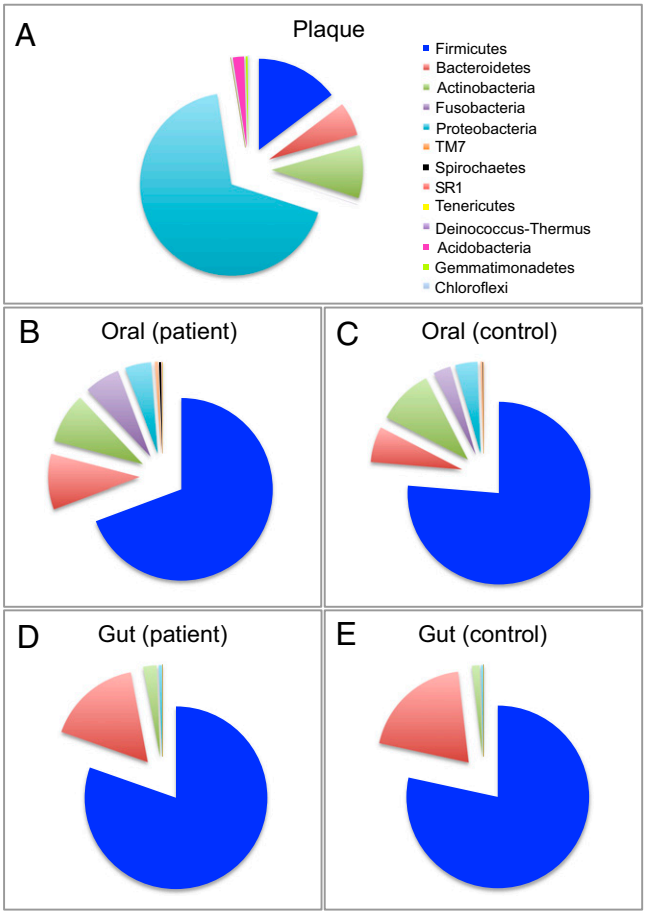

Fig. 2. Mean phylum abundances by body habitat for patients and controls. Plotted values are mean sequence abundances in each phylum for 1,700 randomly selected sequences per sample. (*A*) Atherosclerotic plaque; (*B*) oral cavity, patient; (*C*) oral cavity, control; (*D*) gut, patient; (*E*) gut, control.

bacteria (6% and 3%), Proteobacteria (5% and 4%), and <1%, Spirochaetes, TM7, SR1, and Tenericutes (Fig. 2 *B* and *C*). The NSC analysis did not reveal any species-level OTUs that could discriminate between the healthy and patient oral samples.

To search for correlations between the abundances of OTUs in the oral cavity and markers for cardiovascular disease, we required a minimum sequence count of 100 sequences per genus (across all samples, using 1,700 sequences randomly selected per sample) for inclusion in the analysis. This analysis revealed several medically important OTUs in the oral cavity whose abundances correlated markers for cardiovascular disease (Fig. 4*A*). The abundance of *Fusobacterium* was positively correlated with levels of cholesterol and LDL cholesterol ($\rho = 0.63$ and 0.75, $P = 0.028$ and 0.005, respectively). Interestingly, the abundance of *Streptococcus* was positively correlated to HDL cholesterol and ApoAI levels ($\rho = 0.88$ and 0.70, $P = 0.0001$ and 0.01), whereas the abundance of *Neisseria* was negatively correlated to levels of these two disease markers ($\rho = -0.65$ and -0.74, $P = 0.02$ and 0.005).

Composition of the Gut Microbiota and Its Relation to Atherosclerosis and Disease Markers. Because the fecal microbiota is representative of the gut microbiota (1), we characterized the bacterial diversity of fecal samples to determine whether gut microbiotas differed between patients and controls. Overall, the relative abundances of the phyla were similar between patients and controls, and NSC analysis did not reveal any OTUs whose abundances could differentiate the patients from the controls (Fig. 2). Compared with the oral and atherosclerotic plaque samples, gut samples contained significantly greater abundances of OTUs classified as members of the Lachnospiraceae family, and as the genera *Ruminococcocus* and *Faecalibacterium*. We found the abundances of two OTUs classified as uncharacterized members of Erysipelotrichaceae and Lachnospiraceae families in the gut to be positively correlated with cholesterol ($\rho = 0.69$ and $\rho = 0.79$, $P = 0.009$ and 0.001, respectively) and LDL cholesterol levels ($\rho = 0.67$ and $\rho = 0.70$, $P = 0.012$ and 0.007, respectively). In addition, we observed several other OTUs that correlated with disease markers but did not reach significance (Fig. 4*B*).

Inter- and Intraindividual Comparisons of Atherosclerotic Plaque, Oral, and Gut Microbiotas. One of the main purposes of the study was to search for OTUs shared between oral and atherosclerotic plaque samples, and gut and atherosclerotic plaque samples, within the same individuals. Table 2 summarizes the OTUs that could be found in atherosclerotic plaques and at least one other body habitat in at least two patients. We detected *Veillonella* OTUs in all 13 patients (Table 2). In 11 of the 13 patients that provided oral samples, these OTUs could be detected in both the atherosclerotic

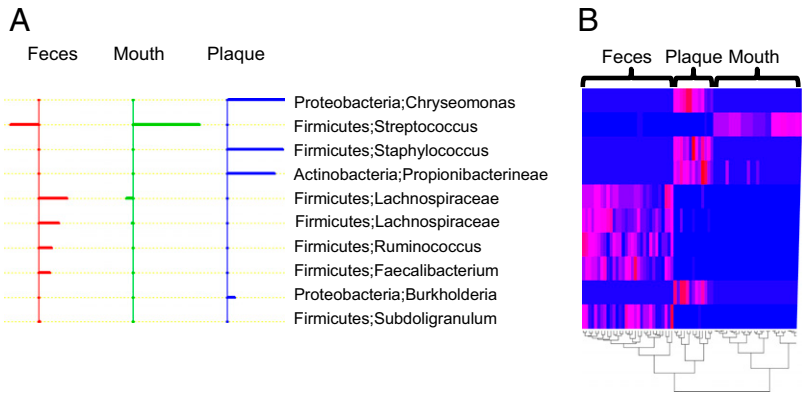

Fig. 3. Members of the microbiota that differ in abundance between the body sites. (*A*) Shrunken differences for the 10 genera accounting for the differences among the three body sites. For each genus listed in center, the direction of the horizontal bars indicates relative overrepresentation (*Right*) and underrepresentation (*Left*), and the length of the bar indicates the strength of the effect. (*B*) Heat map of the abundances of the classifying genera (i.e., those driving differences between body sites). Columns show, for each sample, the abundance data of genera listed in center. The abundances of the genera were clustered using unsupervised hierarchical clustering (blue, low abundance; red, high abundance). The phylum;genus of each of the classifying OTUs is noted.

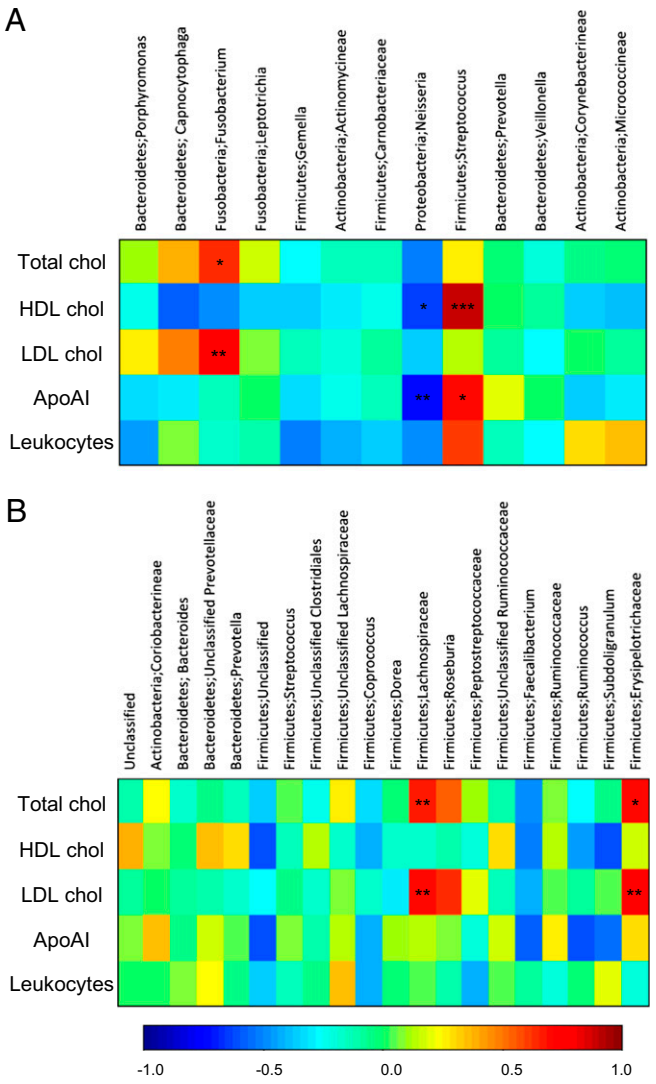

Fig. 4. Correlations between the abundances of different genera and disease markers in oral and fecal samples. Pearson correlation coefficients are represented by color ranging from blue, negative correlation (−1), to red, positive correlation (1). (*A*) OTUs from oral samples; (*B*) OTUs from fecal samples. Significant correlations are noted by $*P < 0.05$; $**P < 0.01$, and $***P < 0.001$.

plaques and the oral cavity samples of the same patients, and in two patients these OTUs were also detected in the gut. *Streptococcus* OTUs had a similar pattern of distribution: In six of the 10 patients, *Streptococcus* OTUs were detected in the oral cavity and atherosclerotic plaques, and in four patients they could also be detected in the gut. Interestingly, the combined abundances of *Veillonella* and *Streptococcus* were correlated in oral cavity and atherosclerotic plaque across patients ($\rho = 0.6$, $P = 0.03$), although when considered singly, the abundances of these bacteria in oral cavity and atherosclerotic plaque were not correlated.

Within individual patients, the atherosclerotic plaque samples contained additional OTUs that were also detected in oral and gut samples. For instance, additional OTUs detected in the atherosclerotic plaque and oral samples of the same individual for at least two patients include *Propionibacterium*, *Rothia*, *Burkholderia*, *Corynebacterium*, *Granulicatella*, *Staphylococcus* and an unclassified OTU belonging to the Betaproteobacteria. In contrast, OTUs detected in the atherosclerotic plaque and gut samples of the same individual for at least two patients include *Bacteroides*, an unclassified member of the Lachnospiraceae, *Bryantella*, *Enterobacter*, an unclassified Enterobacteriaceae, *Ruminococcus*, and OTUs classified as *Subdoligranulum* (Table 2).

Discussion

Here we used barcoded multiplexed-454 pyrosequencing to compare the bacterial composition of the oral, gut, and atherosclerotic plaque microbiotas in patients with atherosclerosis. This approach allowed a relatively comprehensive description of microbial communities associated with atherosclerotic plaques. This study also provides the interindividual comparison of oral, gut, and atherosclerotic plaque samples necessary to identify members of the normal microbiome that may translocate from one body habitat to another where they may contribute to disease. We found that the atherosclerotic plaque microbial communities are like those colonizing other parts of the human body—represented by a core of "signature" species, yet highly diverse and variable between individuals (4). Our findings suggest that the atherosclerotic plaque microbiota may at least in part be derived from the oral cavity and/or the gut. Although we did not observe any overall differences in the oral or gut microbiotas between patients and controls, several taxa correlated with cholesterol and apolipoprotein levels in the plasma.

Chryseomonas (recently reclassified as *P. luteola*) was present in all atherosclerotic plaque samples and may represent a previously unappreciated core member of atherosclerotic plaque communities. This organism has been implicated in endocarditis (24), and its presence in all atherosclerotic plaques studied here raises the possibility that it may directly affect the pathogenesis of atherosclerosis. Although we did not detect it from buccal swabs, it has been described as an inhabitant of the tongue (25). In addition to the diversity described here, another important constituent of atherosclerotic plaque is *Chlamydia* (26). Although not detected in the 16S rRNA gene diversity survey [likely due to suboptimal hybridization of the 16S rRNA gene primers we used to members of this phylum (Chlamydiae) (17)], we confirmed its presence in all plaques by qPCR with *Chlamydia*-specific primers (Table S2).

One intriguing observation was the correlation of combined *Veillonella* and *Streptococcus* abundances in the atherosclerotic plaque samples and in the oral samples across patients. These two genera are known early colonizers of tooth surfaces that interact to form dental plaques: *Streptococcus* produces a preferred fermentation product for *Veillonella* (27). However, because both genera also are common colonizers of the stomach and small intestine (28, 29), we cannot rule out that they may originate from these body sites. Besides these OTUs, we also detected *Rothia*, *Granulicatella*, and *Propionibacterium* in both the oral cavity and the atherosclerotic plaque within the same patients. All three of these genera have been described as normal inhabitants of the healthy oral microbiota (30). Furthermore, they have also been implicated in endocarditis (31–33), and recent data suggest that the transmission of *Granulicatella* from the oral cavity could cause infective endocarditis (31). Interestingly, the oral and atherosclerotic plaque microbiotas have similar levels of diversity (PD). Thus, the pathobiology of the atherosclerotic plaque may be similar to that of dental plaque—a polymicrobial infection characterized by a few key members required for the initiation of disease.

Our analysis also revealed several OTUs shared between the atherosclerotic plaque and the gut, suggesting that bacteria present in the atherosclerotic plaque could also be derived from the distal gut as well as the oral cavity. One mechanism by which bacteria could reach the atherosclerotic plaque is phagocytosis by macrophages at epithelial linings (e.g., the oral cavity, gut, and the lung). Upon phagocytosis, the macrophages become activated, and when they reach the activated endothelium of the atheroma, they leave the blood stream to enter the atheroma and transform into cholesterol-laden foam cells (26). In support of this mechanism, patients with cardiovascular disease have a twofold increase of *C. pneumonia*-infected peripheral blood mononuclear

Table 2. OTUs shared among at least two body sites within the same patient

Consensus lineage (no. of different OTUs with the same consensus lineage)	No. of patients (n = 13)		
	Plaque + feces	Plaque + mouth	Plaque + feces + mouth
Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Veillonella (7)	0	11	2
Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus (17)	0	6	4
Actinobacteria;Actinobacteria;Actinobacteridae;Actinomycetales; Propionibacterineae;Propionibacteriaceae;Propionibacterium (4)	0	8	0
Bacteroidetes;Bacteroidetes;Bacteroidales;Bacteroidaceae;Bacteroides (5)	6	0	0
Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;unclassified Lachnospiraceae(9)	6	0	0
Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Lachnospiraceae Incertae Sedis (4)	5	0	0
Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Bryantella (1)	5	0	0
Actinobacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Micrococcineae; Micrococcaceae;Rothia (2)	0	4	0
Proteobacteria;Gammaproteobacteria;Enterobacteriales; Enterobacteriaceae;Enterobacter (1)	4	0	0
Proteobacteria;Betaproteobacteria;Burkholderiales; Burkholderiaceae;Burkholderia (1)	0	3	0
Proteobacteria;Gammaproteobacteria;Enterobacteriales; Enterobacteriaceae;unclassified Enterobacteriaceae (2)	3	0	0
Actinobacteria;Actinobacteria;Actinobacteridae;Actinomycetales; Corynebacterineae;Corynebacteriaceae;Corynebacterium (2)	0	2	0
Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae;Carnobacteriaceae (2); Granulicatella (1)	0	2	0
Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Staphylococcus (3)	0	2	0
Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminococcus (1)	2	0	0
Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Subdoligranulum (7)	2	0	0
Proteobacteria;Betaproteobacteria;unclassified Betaproteobacteria (1)	0	2	0

Columns at right indicate the number of patients for which the OTUs were found in both plaque and feces; plaque and mouth; or all three sites.

cells compared with controls (34). Furthermore, bacteria are only present in atheromas and not in healthy aortic tissues in mice (35) and have been identified in human atherosclerotic plaques (17). Thus, infected macrophages may specifically target bacteria to atheromas.

An accumulating body of data indicates that the impact of infection on atherosclerosis is related to the total "pathogen burden," i.e., the aggregate number of pathogens infecting an individual (13). Indeed, the observed correlation between the amount of bacterial DNA in atherosclerotic plaques and the leukocyte counts further suggests that atherosclerotic plaque bacterial load determines its inflammatory status and stability. Our findings may thus suggest that bacteria predominantly affect atherosclerosis by activating the innate immune system in the atherosclerotic plaque. Accordingly, atherosclerosis-prone mice deficient in Toll-like receptor (Tlr) 2, Tlr4, or the adapter molecule MyD88 are resistant to the development of atherosclerosis (36–38). Furthermore, polymorphism in Tlr4, which promotes production of proinflammatory cytokines in response to bacterial LPS, is associated with lower levels of proinflammatory cytokines, acute-phase reactants, carotid atherosclerosis, and a smaller intima-media thickness in the common carotid artery (39).

In addition to the shared OTUs between atherosclerotic plaques and oral/gut microbiotas, our analysis revealed that specific components of the oral and gut microbiotas correlated with disease markers. *Streptococcus* was strongly positively correlated with HDL cholesterol and ApoAI (a major component of HDL), whereas *Neisseria* was strongly negatively correlated with these markers. *Fusobacterium* abundance was positively correlated with LDL cholesterol and total cholesterol. Similarly, members of the Erysipelotrichaceae and Lachnospiraceae families in the gut also positively correlated with LDL cholesterol and total cholesterol. Whereas the oral components are unlikely to directly contribute to cholesterol metabolism, they may serve as biomarkers. In contrast, the gut microbiota may directly affect host lipid metabolism (40). Although the exact mechanism remains to be identified, the gut microbiota is essential for bioconversion of cholesterol to bile acids, which are required for cholesterol excretion, as well as cholesterol absorption (41–43). Accordingly, in addition to promoting atherosclerotic plaque inflammatory status, the gut microbiota could also contribute to atherosclerotic disease by modulating host lipid metabolism.

In summary, we detected key bacterial members of dental plaque in atherosclerotic plaques in humans, as well as a novel common member, *Chryseomonas*, in all atherosclerotic plaques. In addition, the atherosclerotic plaques contained numerous bacteria from different phyla. Our findings strongly support the hypothesis that the oral cavity and gut can be sources for atherosclerotic plaque-associated bacteria. Furthermore, members of both the oral and gut microbiotas correlated with disease biomarkers, especially plasma cholesterol. Based on these findings, several lines of inquiry are warranted: investigation into whether oral and/or fecal bacterial community composition can predict atherosclerosis, and how specific bacteria, such as *Chryseomonas*, may contribute to atherosclerosis development and/or progression.

Materials and Methods

Patient and Control Groups. The patient samples were obtained from the Göteborg Atheroma Study Group biobank, which includes carotid endarterectomies from patients who were operated for minor ischemic stroke, transient ischemic attack, or amaurosis fugax as previously described in detail (44). The patients were consecutively included, and completed questionnaires covering previous and current diseases, lifestyle factors, and medication. Before surgery, blood samples were drawn and plasma and serum aliquots were prepared and immediately frozen at −70 °C. The excised endarterectomy specimens were immediately frozen in liquid nitrogen under sterile conditions. The subjects in the control group were obtained from two currently running population-based studies of men and women born 1937–1940 (45–47). These studies were based on screening examinations of randomly selected population-based cohorts. The control subjects were matched to the patient group by sex, and had to fulfill criteria of feeling well and not suffering from past or current cerebrovascular disease.

Each control subject came to the laboratory for information and examinations identical to those performed in the patient group. Mouth swabs were obtained from both groups by a nurse, and the patients were given material and instructions for providing fecal samples. Of the complete sample set, two samples are missing: one oral sample (not obtained) and one atherosclerotic plaque (insufficient material). The study was approved by the ethics committee in Gothenburg. All patients gave written informed consent to participate after oral and written information.

DNA Extraction. Genomic DNA was isolated from 100 mg of feces using the Viogene DNA Extraction Kit. Mouth swabs were soaked in 900 μL lysis buffer for 2 h before DNA was isolated using the same kit. The genomic DNA from the atherosclerotic plaque samples was extracted using the MOBIO PowerSoil DNA Isolation Kit. All samples were extensively homogenized using a bead beater at maximum speed for 3 min. The remaining steps were performed as directed by the manufacturer.

qPCR. The bacterial DNA concentration in plaque was quantified by quantitative PCR (qPCR) according to Sokol et al. (48) and performed under the following conditions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 62 °C for 1 min. A 1:1 mix of purified genomic DNA from *Escherichia coli* and *Lactobacillus reuteri* was used to construct a standard curve in the range of 10 fg to 10 ng. The amount of *Chlamydia* spp. was quantified according to Yoshida et al. (49) and performed under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 56 °C for 1 min. A standard curve was made from genomic DNA from *Chlamydia trachomatis* (DNA from cells infected with *C. trachomatis*, serovar H, strain UW-43/Cx, ATCC, VR-879D) in the range of 10 fg to 10 ng. To ensure that the primers were specific for *Chlamydia*, negative control reactions with *E. coli* and *Bifidobacterium longum* DNA were performed.

PCR Amplification of the V1-2 Region of Bacterial 16S rRNA Genes. 16S rRNA genes were amplified by PCR with a forward primer containing the 454 Life Sciences primer B sequence and the broadly conserved bacterial primer 27F, and a reverse primer containing the 454 Life Sciences primer A sequence, a unique 12-nucleotide error-correcting barcode used to tag each PCR product, and the broad-range bacterial primer 338R (22). PCR reactions were carried out in quadruplicate (gut, oral) or triplicate (atherosclerotic plaques) 20-μL reactions with 0.3 μM forward and reverse primers, with ≈50 ng template DNA and 1× of HotStar Taq Plus Master Mix kit (Qiagen). Thermal cycling consisted of initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s, and extension at 65 °C for 60 s. Replicate amplicons were pooled, purified with Agencourt AMPure Kit (Agencourt), and visualized by electrophoresis using 1.0% agarose gels. Negative extraction control samples were treated similarly, except that no template DNA was added to the PCR reactions; these failed to produce visible PCR products and were not analyzed further.

Amplicon Quantitation, Pooling, and Pyrosequencing. Oral and fecal amplicon DNA concentrations were determined by spectrophotometry (Nanodrop), and atherosclerotic plaque amplicons DNAs were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Amplicons were combined in equimolar ratios into a single tube with a final concentration of 16 ng/μL. Pyrosequencing of oral and fecal samples was carried on a 454 Life Sciences Genome Sequencer FLX instrument at Center for Metagenomic Sequence Analysis at KTH, School of Biotechnology in Stockholm, Sweden, and atherosclerotic plaque samples were sequenced at the Cornell University Life Sciences Core Laboratories Center using Roche/454 Titanium chemistry.

Sequence Analysis. Sequences were denoised and analyzed with the software package Quantitative Insights into Microbial Ecology (QIIME) using default parameters for each step, except where specified (50). Sequences were removed if lengths were <200 nt; contained ambiguous bases, primer mismatches, homopolymer runs in excess of six bases, or uncorrectable barcodes; or lacked the primer. Remaining sequences were assigned to samples according to their barcodes (Table S1). Similar sequences were binned into OTUs using UCLUST (http://www.drive5.com/usearch/), with a minimum pairwise identity of 97%. The most abundant sequence in each OTU was chosen to represent its OTU. Representative sequences from each OTU were aligned using PyNAST (a python-based implementation of NAST in QIIME) (51) and the Greengenes (52) database (corset aligned November 8, 2007) using a minimum percent identity of 75%. Representative sequences were aligned and ChimeraSlayer (http://microbiomeutil.sourceforge.net/) was used for the identification of putative chimeric sequences. From the putatively chimeric sequences in the representative set, we counted the number of total sequences in each of the corresponding OTUs to determine the proportion of total sequences deemed putative chimeras. The lanemaskPH was used to screen out the hypervariable regions (4), and a phylogenetic tree was constructed using FastTree (53). Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier (54) with a minimum support threshold of 60% and the RDP classifier nomenclature. Sequences belonging to OTUs classified as plant chloroplasts were considered contaminants and removed from the analysis.

To compare diversity between samples, we used the weighted and unweighted UniFrac distance metrics (55, 56) using a random sample of 1,700 sequences per sample (one atherosclerotic plaque sample had fewer than 1,500 sequences and was omitted from the analysis). To relate the OTU abundances to patient health data, we considered only OTUs containing at least 100 sequences.

Statistical Analyses. A "nearest-shrunken centroid" classification approach was performed to detect the genera that were particularly representative of each category (atherosclerotic plaque, oral cavity, and gut for the different body habitats, and healthy vs. patient for within oral cavity and gut) (57). The amount of shrinkage was set to minimize the misclassification error. When phyla were used to classify samples, the accuracy of the classification was quite good (overall error rate = 0.097; only 7 of 72 samples were misclassified). All of the misclassified samples were oral cavity samples incorrectly classified as gut. At the genus level, the classification improved (overall error rate = 0.014; one atherosclerotic plaque sample was misclassified as gut). These analyses allowed the identification of phyla/genera whose abundances significantly differed between categories. This analysis was performed using the Prediction Analysis for Microarrays (PAM) package within R software.

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