ORIGINAL ARTICLE

Bacteria and bacterial DNA in atherosclerotic plaque and aneurysmal wall biopsies from patients with and without periodontitis

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Background: Several studies have reported an association between chronic periodontitis (CP) and cardio-vascular diseases. Detection of periodontopathogens, including red complex bacteria (RCB), in vascular lesions has suggested these bacteria to be involved in the pathogenesis of atherosclerosis and abdominal aortic aneurysms.

Objective: In this study, we investigate bacteria and their DNA in vascular biopsies from patients with vascular diseases (VD; i.e. abdominal aortic aneurysms, atherosclerotic carotid, and common femoral arteries), with and without CP.

Methods: DNA was extracted from vascular biopsies selected from 40 VD patients: 30 with CP and 10 without CP. The V3-V5 region of the 16S rDNA (V3-V5) was polymerase chain reaction (PCR)-amplified, and the amplicons were cloned into *Escherichia coli*, sequenced, and classified (GenBank and the Human Oral Microbiome database). Species-specific primers were used for the detection of *Porphyromonas gingivalis*. In addition, 10 randomly selected vascular biopsies from the CP group were subjected to scanning electron microscopy (SEM) for visualization of bacteria. Checkerboard DNA–DNA hybridization was performed to assess the presence of RCB in 10 randomly selected subgingival plaque samples from CP patients.

Results: A higher load and mean diversity of bacteria were detected in vascular biopsies from VD patients with CP compared to those without CP. *Enterobacteriaceae* were frequently detected in vascular biopsies together with cultivable, commensal oral, and not-yet-cultured bacterial species. While 70% of the subgingival plaque samples from CP patients showed presence of RCB, only *P. gingivalis* was detected in one vascular biopsy. Bacterial cells were seen in all 10 vascular biopsies examined by SEM.

Conclusions: A higher bacterial load and more diverse colonization were detected in VD lesions of CP patients as compared to patients without CP. This indicated that a multitude of bacterial species both from the gut and the oral cavity, rather than exclusively periodontopathogens, may be involved as additional risk factors in the pathogenesis of VD.

Keywords: *chronic periodontitis*; *vascular disease*; *16S rDNA*; Porphyromonas gingivalis

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Chronic periodontitis (CP) and atherosclerotic and abdominal aortic aneurysmal vascular disease (VD) are highly prevalent, chronic inflammatory conditions with complex and multifactorial etiology. CP is initiated by the accumulation of dental plaque with a subsequent gingival inflammatory immune response that may progress to the destruction of connective tissue and alveolar bone (1). Atherosclerosis is characterized by initial endothelial injury followed by invasion of macrophages, accumulation of lipids, and calcification of the subendothelial layer, and can cause disabling and life-threatening conditions such as myocardial infarction and stroke (2). The mechanisms causing the initiation and progression of the abdominal aortic aneurysm are

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poorly understood. Inflammation, matrix degradation, and thrombosis formation are important features of the disease, which is a significant cause of morbidity and mortality in the adult population (3).

Traditional risk factors such as smoking, hypercholesterolemia, hypertension, and diabetes mellitus cannot fully account for the overall incidence of VD (4). Chronic bacterial infections such as periodontitis are suggested to be additional risk factors (5–8).

The gingival epithelium is part of the innate immune system acting as a physical barrier against invasion of oral bacteria. In the oral cavity, approximately 700 different major bacterial species have been identified by 16S rDNA sequencing, and more than 400 species are identified from periodontal pockets (9). In sites with active periodontal disease, the epithelial barrier is ulcerated and discontinuous and may allow dissemination of oral bacteria into the underlying connective tissue and capillaries, and eventually into the systemic circulation. Transient bacteremias in patients with periodontitis have been detected after regular activity such as tooth brushing and following periodontal treatment (10–12). Direct hematogenous spreading of oral bacteria and invasion of endothelial and smooth muscle cells are considered to be the main mechanisms of the association between periodontitis and cardiovascular diseases. In addition, oral bacteria may also reach the arterial walls by invading phagocytic cells (6, 13).

Several studies have identified periodontopathogens such as *Porphyromonas gingivalis* and *Treponema denticola* in atherosclerotic lesions (14–17). *In vitro* studies and *in vivo* animal studies have shown that bacteria associated with periodontal disease, such as *P. gingivalis*, may contribute to vascular inflammation by several mechanisms such as activation of toll-like receptors, increasing the production of pro-inflammatory mediators, and the expression of cell-surface adhesion molecules, causing apoptosis of vascular cells, and triggering of the coagulation cascade (6, 13). Using quantitative polymerase chain reaction (PCR), Gaetti-Jardim et al. demonstrated that periodontopathic bacterial DNA represented 47.3% of the total bacterial DNA found in atheromatous samples from patients with periodontitis and 7.2% of the total bacterial DNA detected in atheromas from periodontally healthy subjects (18). Ott et al. (19) reported an overall high bacterial diversity with more than 50 bacterial species in the coronary atherosclerotic tissue using 16S rDNA clone libraries. These and other studies suggest that a multitude of bacterial species may contribute to atherogenesis and abdominal aortic aneurysm formation (5, 20, 21).

The objective of this case-control study was to investigate the bacterial diversity in atherosclerotic plaque and aneurysmal wall biopsies from patients with and without CP by sequencing 16S rDNA clone libraries.

In addition, the presence of *P. gingivalis* in VD biopsies from CP patients was investigated using species-specific PCR and subsequent sequencing. Scanning electron microscopy (SEM) was used to visualize bacterial cells in VD lesions.

Material and methods

Participants
This study included a total of 77 patients with VD who were recruited at the Department of Vascular Surgery, Oslo University Hospital, Aker, Oslo, Norway. They were scheduled for vascular surgery, including abdominal aortic aneurysm repair and carotid or femoral arterial endarterectomy. Clinical oral examinations were performed at the hospital ward to determine the periodontal status of the patient.

The patients were categorized into two groups according to their clinical periodontal status. The first group (VD with CP, $n = 30$) were patients undergoing treatment for VD and who were diagnosed with CP. The second group (VD without CP, $n = 47$) were patients undergoing treatment for VD and who were assessed without periodontitis.

The patients' medical records were obtained for general health information. Questions on ethnicity and smoking behavior were self-reported. Written informed consent was obtained from all participants. This study was approved by the Regional Ethical Committee (REK Sør, NO. 08/322b) and was in accordance with the Helsinki declaration of 1975, as revised in 1983. This study has been registered at ClinicalTrials.gov (ID. NCT01358630).

Inclusion criteria
The patients in both groups were diagnosed and treated according to standard procedures at the Department of Vascular Surgery, Oslo University Hospital, Aker. Diagnosis of CP was based on the classification system of the American Academy of Periodontology, established in 1999 at the International Workshop for Classification of Periodontal Diseases and Conditions (22). Clinical periodontal status was assessed by periodontal pocket probing depth and bleeding on probing. The measurements were done at the mesial, buccal, distal, lingual, and palatal surfaces of all teeth. Subjects who had at least four sites with a probing depth ≥ 5 mm and bleeding on probing were categorized as having CP. All periodontal examinations were done by an experienced dentist. No subject had received periodontal treatment within the last 6 months or taken antibiotics within the past month.

Sample collection and DNA extraction
Vascular tissue biopsies and subgingival plaque samples were collected for extraction of genomic DNA and checkerboard DNA–DNA hybridization analysis, respectively. The vascular biopsies were collected under surgical

treatment from the walls of aneurysms and during excision of intravascular plaques in carotid or common femoral arteries. The biopsies were immediately transferred from bedside to a sterilized container that was brought on ice to the laboratory and stored at -80°C. The collection of the biopsies and further processing in the laboratory were performed under strict aseptic conditions.

Extraction of genomic DNA was done by using the Masterpure Complete DNA Purification Kit (Epicentre Biotechnologies, Madison, WI), according to the manufacturer's extraction protocol for tissue samples with some modifications.

In order to make a representable selection of the biopsies for DNA extraction and assist cell lysis, the vascular biopsies were mechanically homogenized. Proteinase K (100 µg) was used for cell lysis treatment. After the samples were treated with RNase A (5 µg) and protein-precipitated, they were kept in 0.5 M NaCl. Further, they were purified with (1:1 v/v) phenol–chloroform (VWR International AS, Oslo, Norway) and centrifuged in Phase Lock Gel tubes (5Prime, Gaithersburg, MD). The aqueous phase was treated twice with chloroform iso-amyl alcohol (1:1 v/v) (AppliChem GmbH, Darmstadt, Germany). DNA was precipitated with 100% ethanol (Kemetyl, Vestby, Norway) in 0.3 M sodium acetate (AppliChem GmbH). The pellet was washed twice with 75% ethanol, resuspended in $1 \times$ TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at -20°C.

Negative and positive control reactions were performed for the tissue homogenization and DNA extraction methods.

The subgingival plaque samples were collected from four periodontal sites for each subject. In patients with CP, the deepest pockets with bleeding on probing were chosen, while in patients without CP, four sites were chosen randomly. After removal of supragingival plaque, isolation, and drying of sample sites with cotton rolls, subgingival plaque was collected by using sterile Gracey curettes. Samples were pooled and transferred to tubes containing 300 µl TE buffer.

PCR amplification of bacterial DNA

Universal bacterial primers were used for 16S rDNA amplification under standardized conditions with forward primer: E334F 5′-CCAGACTCCTACGGGAGGCAGC-3′ and reverse primer: E939R 5′-CTTGTGCGGGCCC CCGTCAATTC-3′ (23). These primers cover the hypervariable region V3-V5 of the 16S rRNA gene, have high specificity to bacterial sequences, and low match to Eukarya and Archaea sequences (23). PCRs were performed with Accuprime Supermix II (Invitrogen, Carlsbad, CA) at an annealing temperature of 69°C and a total of 32 cycles.

Species-specific PCR assays and *fimA* genotyping were applied for the identification of the periodontopathogen *P. gingivalis* in vascular biopsies from patients with CP. The previously reported primers for *P. gingivalis* 16S rRNA and *fimA* gene (24, 25) were used (Table 1). PCRs were performed with OneTaq 2X Master Mix with standard buffer (New England Biolabs, Beverly, MA) and annealing temperatures as given in Table 1.

Each set of experiments included negative controls with sterile molecular grade water instead of template DNA and positive controls containing purified DNA from *Veillonella dispar*, *P. gingivalis* strains for *fimA* type I (ATCC 33277T) and *fimA* type II (A7A1-28). The PCR products were detected by 1% agarose gel electrophoresis. Gels were stained with ethidium bromide, using 1Kb Plus DNA Ladder (Invitrogen).

Cloning and sequencing

The universal 16S rDNA PCR amplicons (primers E334/ E939) were ligated into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen) and transformed into competent *Escherichia coli* TOP10 cells according to the manufacturer's instructions. Ninety-six colonies were collected from each sample to make a representative library of the PCR products, which were stored in the TE buffer at -20°C until further processing. After PCR amplification of the inserts with M13 forward primer, 5′-GTA AAA CGA CGG CCA G-3′, and M13 reverse primer, 5′-CAG GAA ACA GCT ATG AC-3′, the PCR

Table 1. Porphyromonas gingivalis 16S rDNA and *fimA* gene-specific primers used in this study

Primer set	Sequence (5′-3′)	Annealing (°C)	Amplicon size (bp)
P. gingivalis 16S rDNA	TGT AGA TGA CTG ATG GTG AAA ACC ACG TCA TCC CCA CCT TCC TC	60°C	197
M11/M12 *fimA*	AATCTGAACGAACTGCGACGCTAT CTCCCTGTATTCCGAATATAGAC	55°C	~1,300
Type I *fimA*	CTG TGT GTT TAT GGC AAA CTT C AAC CCC GCT CCC TGT ATT CCG A	58°C	392
Type II *fimA*	ACA ACT ATA CTT ATG ACA ATG G AAC CCC GCT CCC TGT ATT CCG A	50°C	257

products were purified by Exosap-IT (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. Purified amplicons were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the M13 forward primer.

The *P. gingivalis*-specific PCR amplicons were sequenced using the BigDye terminator v1.1 Cycle Sequencing Kit according to the manufacturer's instructions and the respective forward primers (Table 1). Sequence reactions were run on an ABI Prism 3,730 DNA analyzer (Applied Biosystems).

Data analysis of the sequences

Sequence trimming and quality check were performed with the Sequencher 5.0 program (Gene Codes Corp., Ann Arbor, MI). The UCHIME program (26) at the mothur platform (27) was used for chimeric check. After the elimination of suspected chimeric sequences, the 16S rDNA sequences (~ 500 bp) were used to determine bacterial identity. The sequences were aligned by using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (28).

For identification of closest relatives, the consensus sequences were compared with known sequences in the GenBank databases using the NCBI BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST/) and Human Oral Microbiome Database (HOMD; http://www.homd.org/) (29).

Checkerboard DNA–DNA hybridization

Ten randomly selected subgingival plaque samples from VD patients with CP were assessed for the presences of red complex bacteria (RCB) using checkerboard DNA–DNA hybridization. NaOH (100 µl of 0.5 M) was added to 100 µl of subgingival plaque samples in TE buffer and vortexed. After boiling for 10 min, 800 µl of 5 M ammonium acetate was added to neutralize the samples. The DNA was then applied into the extended slots of a Minislot-30 apparatus (Immunetics, Cambridge, MA) with a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN). The membrane had two lanes with DNA standards equivalent to 10^5–10^6 cells per target species tested. The membranes were hybridized with 20 digoxigenin labeled whole genome probes of species significant for the subgingival microbiota, including RCB (Table 2) (30). DNA from the type species was used as probes.

Scanning electron microscopy

Ten randomly selected vascular biopsies from VD patients with periodontitis were selected at random for SEM. The biopsies were cut into blocks of approximately 5×5 mm. Tissue blocks were fixed in 2.5% glutaraldehyde or 0.1 mol/L Sorensen phosphate buffer, and stored at 4°C until processing. After dehydration in ethanol, the blocks were critically point-dried with carbon dioxide.

Table 2. Bacterial species detected by DNA–DNA hybridization (checkerboard analysis) in subgingival dental plaque samples from patients with atherosclerotic and abdominal aortic aneurysmal vascular disease and chronic periodontitis

	Detection frequency in subgingival dental plaque samples ($n = 10$)
Fusobacterium nucleatum ssp. *vincentii*	1.0
Eubacterium saburreum	1.0
Prevotella nigrescens	0.9
Actinomyces viscosus	0.8
Actinomyces gerencseriae	0.8
Actinomyces israelii	0.7
Streptococcus intermedius	0.7
Porphyromonas gingivalis	0.7
Fusobacterium nucleatum ssp. *polymorphum*	0.6
Fusobacterium nucleatum ssp. *nucleatum*	0.6
Tannerella forsythia	0.6
Aggregatibacter actinomycetemcomitans	0.4
Treponema denticola	0.4
Porphyromonas endodontalis	0.4
Campylobacter rectus	0.2
Treponema socranskii ssp. *socranskii*	0.2
Prevotella melaninogenica	0.2
Eikenella corrodens	0.2
Parvimonas micra	0.1
Prevotella intermedia	0.0

The specimens were finally sputter-coated with gold or palladium in a vacuum evaporator and examined in a scanning electron microscope (XL 30 ESEM; Philips, Eidenhoven, The Netherlands) (31).

Statistical analyses

The statistical analyses were performed using the SPSS software (IBM SPSS Statistic version19). Independent samples *t*-tests were used for comparisons of species diversity and bacterial load between the two groups. For comparisons of demographic data between the two groups, independent *t*-test and Fisher's exact test were performed. A statistically significant difference was defined with $p < 0.05$.

Results

This study included a total of 77 patients with VD. They were categorized into two groups according to their clinical periodontal status. The first group (VD with CP) were patients undergoing treatment for VD and who

were diagnosed with CP ($n = 30$, mean age $= 66.9$, standard deviation (SD) $= 7.9$, range: 51.3–85.0, male $= 25/30$ (83%)). The second group (VD without CP) were patients undergoing treatment for VD and who were assessed without periodontitis ($n = 47$, mean age $= 70.9$, SD $= 9.7$, range: 45.7–84.8, male $= 37/47$ (79%)). Vascular biopsies from all 30 VD patients with CP and 10 randomly selected VD patients without CP were further analyzed for detection of 16S rDNA sequences. In total, 27 biopsies from abdominal aortic aneurysmal walls, 9 from carotid, and 4 from common femoral atherosclerotic plaque were examined. Demographic data of the two study groups are presented in Table 3. The patients with CP had significantly higher number of periodontal pockets with probing depth ≥ 5 mm ($p < 0.01$). They also had a significantly higher pack-year smoking history ($p < 0.01$). There was no significant difference in terms of age, gender, diabetes, body mass index, and number of teeth between the two groups.

After discarding incomplete sequences, a total of 2,638 sequences from VD patients with CP and 859 sequences from VD patients without CP were analyzed. A sequence similarity threshold of ≥ 97 and $\geq 99\%$ were applied for identification at the genus and species level, respectively (32). Sequences with similarity lower than 97% were ranked as unclassified and were excluded (listed according to the closest relative in Supplementary file *SA*). After optimization of the PCR, weak PCR bands were detected in the negative PCR controls ($\sim 50\%$) and were subsequently sequenced. These background sequences (Supplementary file *SB*) as well as eukaryote and chimeric sequences were also discarded from the sequences obtained from the vascular samples and further analyzed. A final total of 38 out of 40 (95%) vascular samples with bacterial DNA were subjected to further analysis.

After discarding unwanted sequences, the final fraction of bacterial sequences, reflecting overall bacterial load, obtained from the vascular biopsies in patients with CP was in average $68 \pm 23\%$ (range: 0–98%) at an individual level. In patients without CP, this fraction was significantly lower with an average of $25 \pm 26\%$ (range: 0–84%; $p < 0.01$). No significant difference in bacterial load was found comparing intravascular plaque versus aneurysmal vascular biopsies in any group.

Eighty-four different bacterial taxa were detected from a total of 1,808 sequences from the VD patients with CP (Table 4). From the VD patients without CP, 18 different taxa were identified from a total of 210 sequences

Table 3. Demographic data of the study groups with atherosclerotic and abdominal aortic aneurysmal vascular disease (VD), with and without chronic periodontitis (CP)

	VD with CP	VD without CP	
	$n = 30$	$n = 10$	p
Age			
Mean $\pm$ SD	66.9 ± 7.9	68.4 ± 10.2	0.66
Median (range)	66.6 (51.3–85.0)	69.1 (48.9–83.6)	
Gender (n)			1.00*
Male	25	8	
Female	5	2	
Teeth (n)			
Mean $\pm$ SD	19.1 ± 7.0	19.6 ± 9.2	0.87
Median (range)	20 (4–27)	23.5 (2–28)	
Periodontal pocket probing depth ($n \geq 5$ mm)			
Mean $\pm$ SD	17.9 ± 11.8	0.5 ± 0.97	<0.01
Median (range)	15.5 (4–55)	0 (0–3)	
Diabetes (n)			0.15*
	1	2	
Smoking (n)			
Non-smokers	1	3	
Current or previous smokers	29	7	
Pack-year Mean $\pm$ SD	36.4 ± 20.3	19.4 ± 11.4	<0.01
Pack-year Median (range)	34.2 (4.9–88.0)	17.3 (5.2–51.3)	
BMI (kg/m^2)			
Mean $\pm$ SD	26.4 ± 4.2	27.7 ± 4.2	0.43
Median (range)	26.4 (14.2–35.3)	26.3 (22.6–37.0)	

*Based on a Fisher's exact test.

Table 4. Bacterial taxa identified in atherosclerotic plaque and abdominal aortic aneurysmal wall biopsies from patients with chronic periodontitis

	16S rDNA sequence frequency (total $n = 1{,}808$)		*n* vascular biopsies positive (total $n = 30$)
Closest relative	n	%	
1. *Serratia* sp.*	251	13.9	15
2. *Propionibacterium acnes***	229	12.7	21
3. *Pseudomonas* sp.**	179	9.9	11
4. *Acinetobacter* sp.**	108	6.0	11
5. *Phenylobacterium* sp.	79	4.4	8
6. Uncultured *Firmicutes* bacterium	79	4.4	10
7. *Flavobacterium* sp.	60	3.3	6
8. Uncultured unclassified bacterium****	44	2.4	7
9. Uncultured *Actinobacterium*	31	1.7	3
10. *Bacillus* sp.***	30	1.7	2
11. *Staphylococcus petenkoferri***	28	1.5	1
12. *Chitinophagaceae* bacterium	28	1.5	1
13. *Corynebacterium* sp.**	27	1.5	5
14. *Klebsiella* sp.*	27	1.5	5
15. *Streptococcus* sp.**	25	1.4	4
16. *Dietzia* sp.	24	1.3	3
17. *Bradyrhizobium* sp.	23	1.3	3
18. *Enterococcus* sp.	22	1.2	3
19. Uncultured *Rhodobacteraceae* bacterium	22	1.2	3
20. *Microbacterium* sp.	21	1.2	4
21. *Moraxella osloensis*	21	1.2	2
22. Uncultured *Geobacter* sp.	20	1.1	1
23. *Staphylococcus epidermidis***	19	1.1	2
24. *Ralstonia picketti***	18	1.0	2
25. *Burkholderia* sp.**	14	0.8	5
26. *Pedobacter* sp.	14	0.8	2
27. *Alishewanella* sp.	13	0.7	2
28. *Stenotrophomonas maltophilia*	13	0.7	2
29. *Kytococcus sedentarius*	12	0.7	1
30. *Oxalobacteraceae* bacterium	12	0.7	1
31. *Sediminibacterium* sp.	12	0.7	1
32. *Acinetobacter bereziniae***	11	0.6	2
33. *Afipia* sp.	11	0.6	2
34. *Alloprevotella* sp.	11	0.6	1
35. *Capnocytophaga sputigena*	11	0.6	1
36. *Micrococcus* sp.	11	0.6	1
37. Uncultured *Flavobacterium* sp	11	0.6	1
38. *Hymenobacter roseosalivarius***	10	0.6	1
39. *Rhizobium* sp.	10	0.6	1
40. Uncultured *Oxalabacteriacae* bacterium	10	0.6	1
41. *Caulobacter* sp.	9	0.5	1
42. *Friedmanniella* sp.	9	0.5	2
43. *Nocardioides* sp.	9	0.5	3
44. *Staphylococcus* sp.**	9	0.5	1
45. *Adhaeribacter* sp.	8	0.4	1
46. *Blastococcus* sp.	8	0.4	1
47. *Streptomyces* sp.	8	0.4	1
48. *Massilia* sp.	7	0.4	2

Table 4 (*Continued*)

Closest relative	16S rDNA sequence frequency (total $n = 1,808$)		n vascular biopsies positive (total $n = 30$)
	n	%	
49. *Ornithinimicrobium* sp.	7	0.4	1
50. Uncultured *Gallionella* sp.	7	0.4	1
51. *Acidovorax* sp.	6	0.3	3
52. *Actinomyces oris*	6	0.3	2
53. *Carnobacterium* sp.	6	0.3	1
54. *Peptoniphilus tyrrelliae*	6	0.3	1
55. *Psychrobacter* sp.	6	0.3	2
56. *Rhodoplanes* sp.	6	0.3	1
57. *Roseinatronobacter* sp.	6	0.3	1
58. Uncultured beta *Proteobacteria*	6	0.3	1
59. *Brevundimonas* sp.	5	0.3	1
60. *Comamonas* sp.	5	0.3	1
61. *Facklamia tabacinasalis*	5	0.3	2
62. *Thermoanaerobacterium* sp.	5	0.3	1
63. *Veillonella dispar*	5	0.3	1
64. Uncultured *Chitinophaga* sp.	5	0.3	1
65. Uncultured *Elusimicrobium* sp.	5	0.3	1
66. *Aerococcus* sp.	4	0.2	1
67. *Porphyromonas gingivalis*	4	0.2	1
68. *Rhodococcus* sp.	4	0.2	1
69. *Tepidimonas* sp.	4	0.2	1
70. Uncultured delta *Proteobacterium*	4	0.2	1
71. *Agrococcus terreus*	2	0.1	1
72. *Alphaproteobacteria*	2	0.1	1
73. *Aquabacterium* sp.	2	0.1	1
74. *Delftia acidovorans*	2	0.1	1
75. *Lysinibacillus* sp.	2	0.1	1
76. *Massilia haematophila*	2	0.1	1
77. *Roseococcus* sp.	2	0.1	1
78. *Roseomonas aquatica*	2	0.1	1
79. *Staphylococcus aureus***	2	0.1	1
80. *Cellulomonas* sp.	1	0.1	1
81. *Corynebacterium afermentans***	1	0.1	1
82. *Streptococcus sanguinis***	1	0.1	1
83. *Streptococcus salivarius***	1	0.1	1
84. *Undibacterium* sp.	1	0.1	1

The different taxa were identified by NCBI BLAST analysis using a similarity threshold of $\geq 97\%$ for identification

Possible overlap with bacterial taxa identified in vascular biopsies from VD patients without CP at *family, **genus, ***species, and ****unranked levels.

Bacterial taxa underlined are listed among the human oral microbial taxa in Human Oral Microbiome Database (HOMD).

(Table 5). The relative abundance of the different taxa detected in vascular biopsies from patients with and without CP is presented in Tables 4 and 5, respectively. The mean diversity of bacterial taxa detected from the vascular biopsies was significantly higher in patients with CP ($n = 30$), average of 7.1 ± 3.2 (range: 0–12), compared to those without CP ($n = 10$), average of 2.8 ± 1.6 (range: 0–5; $p < 0.01$). No significant difference in mean bacterial diversity was found comparing intravascular plaque versus aneurysmal vascular biopsies in any group.

Patients with CP displayed a wide variety of bacteria identified at the genus level from their vascular biopsies, including some known oral bacterial taxa (e.g. *Streptococcus* spp., *Prevotella* sp., and *Capnocytophaga* sp.).

Table 5. Bacterial taxa identified in atherosclerotic plaque and abdominal aortic aneurysmal wall biopsies from patients without chronic periodontitis

Closest relative*	16S rDNA sequence frequency (Total $n = 210$)		*n* vascular biopsies positive (Total $n = 10$)
	n	%	
1. *Bacillus idriensis*	**44**	**21.0**	**1**
2. *Pseudomonas* sp.	**35**	**16.7**	**5**
3. *Delftia* sp.	**20**	**9.5**	**1**
4. *Ralstonia* sp.	**18**	**8.6**	**2**
5. *Corynebacterium* sp.	**15**	**7.1**	**1**
6. *Propionibacterium acnes*	**15**	**7.1**	**3**
7. *Mycobacterium* sp.	12	5.7	1
8. *Salinicoccus* sp.	10	4.8	1
9. *Burkholderia* sp.	**9**	**4.3**	**2**
10. *Hymenobacter* sp.	**6**	**2.9**	**2**
11. *Uncultured unclassified bacteria*	**6**	**2.9**	**1**
12. *Streptococcus* sp.	**5**	**2.4**	**2**
13. *Staphylococcus* sp.	**4**	**1.9**	**1**
14. Uncultured *Bacteroidales* bacterium	3	1.4	1
15. *Paracoccus* sp.	3	1.4	1
16. *Acidobacteria* bacterium	2	1.0	1
17. *Enterobacteriaceae* bacterium	**2**	**1.0**	**1**
18. *Acinetobacter* sp.	**1**	**0.5**	**1**

The different taxa were identified by NCBI BLAST analysis using a similarity threshold of $\geq 97\%$ for identification.

Possible overlap with bacterial taxa identified in vascular biopsies from VD patients with CP is highlighted in bold.

Bacterial taxa underlined are listed among the human oral microbial taxa in Human Oral Microbiome Database (HOMD).

Members of the *Enterobacteriaceae* family (e.g. *Serratia* sp. and *Klebsiella* sp.) were also frequently identified in this group (Table 4). *Pseudomonas* sp. and *Propionibacterium acnes* were abundant in both groups of patients. *Streptococcus*, *Staphylococcus*, and *Acinetobacter* spp. were also detected in both groups and were among the most prevalent species in the VD group with CP. In total, 15% and 4.3% of the sequences were identified as not-yet-cultured bacterial spp. in the VD group with and without CP, respectively. Environmental species, e.g. *Phenylobacterium* and *Bradyrhizobium* spp., were also identified.

Using universal bacterial 16S rDNA PCR, *P. gingivalis* was detected in the vascular biopsy from only one VD patients with CP. No other members of the RCB were detected from the vascular biopsies in either group. None of the 30 vascular biopsies from VD patients with CP were positive for *P. gingivalis* using the specific primers listed in Table 1. Weak PCR bands were detected in the vascular samples using the *fimA* II primer set. Sequencing these bands showed non-specific binding of primers to human DNA. However, checkerboard analysis revealed presence of one to three members of the RCB species in 7 out of 10 subgingival plaque samples from the CP patients (Table 2).
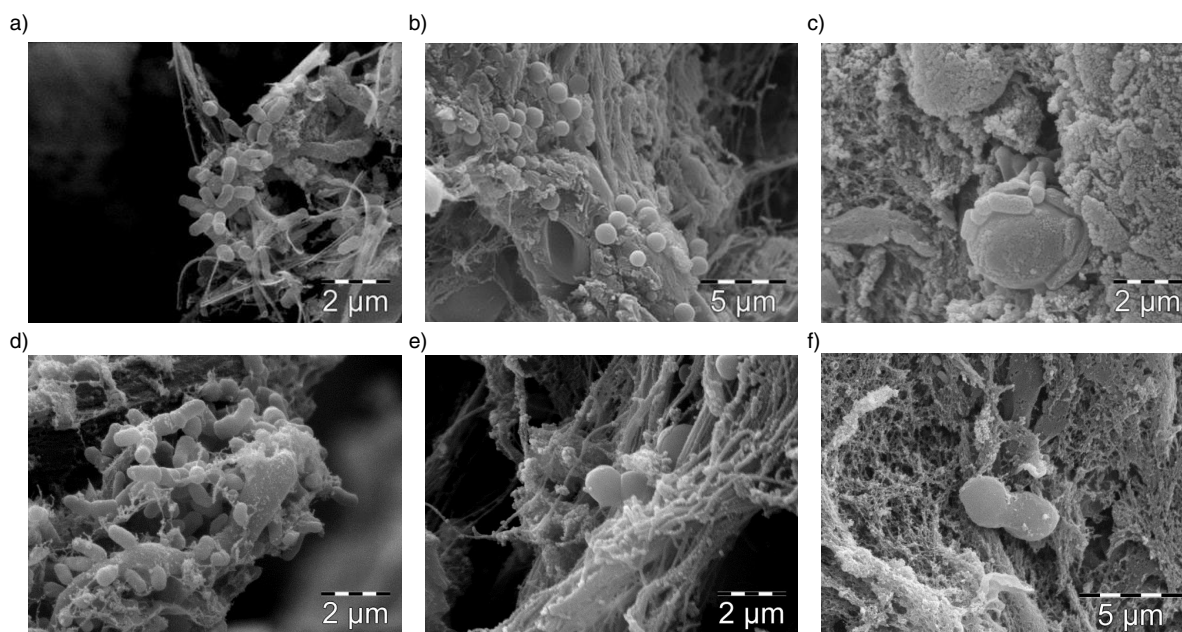
Ten vascular biopsies from VD patients with CP were randomly selected for SEM. Cocci and rod-shaped bacterial cells of different sizes were seen in all biopsies (Fig. 1a–f). Bacteria were observed at the surface of the intravascular plaque lining the arterial lumen entangled in a meshwork of delicate fibers and plaque remnants. Several preparations from each biopsy were inspected in order to visualize bacterial cells. Most often, the bacterial cells were found coaggregated as micro-colonies at a distance from each other. The coaggregated bacteria in each location were most often of uniform morphology. Apparent bacterial cell division with invagination of the cell membrane was seen in some of the SEM images.

Discussion

Bacterial DNA was detected in 95% of the vascular biopsies, and most biopsies contained DNA from multiple bacterial species, pointing to a less specific relationship between single species and VD. These findings are supported by recent studies suggesting multiple bacterial species being involved in cardiovascular diseases (5, 21).

Fig. 1. Scanning electron micrographs of bacteria in vascular biopsies from patients with periodontitis. (a) Area of aneurysmal wall with bacteria entangled in meshwork of delicate fibers. (b) Area of aneurysmal wall with bacteria entangled in meshwork of delicate fibers and remnants of intravascular plaque on aneurysmal wall. (c) Microorganisms (rods) with remnants of intravascular plaque on aneurysmal wall. (d) Area of aneurysmal wall with bacteria entangled in meshwork of delicate fibers and remnants of intravascular plaque on aneurysmal wall. (e, f) Area of aneurysmal wall with coccus-shaped bacteria entangled in meshwork of delicate fibers and remnants of intravascular plaque on aneurysmal wall. Apparently, division of coccus-shaped bacteria is occurring.

We detected a significantly higher mean diversity and a higher load of bacteria in the vascular biopsies from the CP group as compared to the group without CP. In patients with CP, the defective epithelial barrier of the periodontal pockets together with a high load and diversity of bacteria in the subgingival plaque make it conceivable that the oral 'gateway' is contributing to the bacterial composition detected in VD biopsies. Bacterial translocation and transient bacteremia are caused by dental procedures, oral infections, and may even follow tooth brushing and chewing (10). Blood-borne microorganisms are usually cleared by the immune system within minutes. However, favorable conditions may allow colonization at a given site, as seen in cases of infective endocarditis (33).

Even though RCBs were detected at high frequencies in the subgingival plaque samples, only *P. gingivalis* was identified from only one vascular biopsy from a patient with CP. In addition, we used *P. gingivalis*-specific primers; however, none of these primers yielded positive detection of *P. gingivalis* in our VD biopsies. Similarly, several other studies, using species-specific PCR, nested PCR and hybridization detection techniques, also reported no detection of RCB in atherosclerotic carotid lesions, despite the presence of one or more periodontopathogens in the patients' subgingival plaque sam-

ples (34–36). A possible explanation to the negative or sparse detection of RCB in VD biopsies may be that, in contrast to commensal bacteria, 'more antigenic' microbes such as the RCB are readily recognized by the immune system and eliminated from the circulation. In a study by Nakano et al. (37), *Streptococcus mutans* strains serotype *k* survived in blood for a longer time due to lower antigenicity as compared to more cariogenic strains. In addition, a variation in ability to invade cardiovascular cells has been demonstrated among different *P. gingivalis* strains (38). Similar invasive heterogeneity is reported for *Aggregatibacter actinomycetemcomitans* and *S. mutans* strains (39, 40). Individual differences in harboring invasive strains may be an alternative explanation of the discrepancies in the detection of RCB in vascular tissue reported in the literature (11).

Nevertheless, many studies have reported the presence of periodontopathogens in atherosclerotic lesions from aorta, coronary, carotid, and femoral arteries (14, 16, 17, 41), although with variable frequency (11, 36). These variations may be due to methodological differences, e.g. origin of specimens, DNA extraction techniques, the use of specific or universal primers, and PCR conditions (14, 35). However, even by using similar PCR-based techniques with the same specific primers, various detection rates for RCB (0–32% for *P. gingivalis*) in peripheral

atherosclerotic biopsies from patient with CP have been reported (41, 42). With the *P. gingivalis fimA* II primer set previously used for *P. gingivalis* detection in cardiovascular specimen (43), weak PCR bands were detected in some of our vascular samples. Sequencing of these bands showed non-specific binding of primers to human DNA. These findings together with the human sequences identified, using bacterial-specific 16S rDNA primers, emphasize the importance of identifying PCR products by sequencing in studies involving specimens where bacterial DNA is sparse. Using nested PCR and sequencing, Fiehn et al. (44) reported a detection rate of 4.17% for *P. gingivalis* in carotid and femoral atherosclerotic biopsies. None of the other members of the RCB were detected. Ott et al. (19) found an overall high bacterial diversity with more than 50 bacterial species in coronary atherosclerotic tissue using 16S rDNA clone libraries; however, they did not detect any members of RCB. By using 454 high-throughput sequencing, Koren et al. (45) also could not confirm the presence of DNA from periodontopathogens in carotid artery biopsies. Sparse finding of RCB in abdominal aneurysmal samples (Tannerella forsythia in 1 out of 10 samples) was also reported in a study by Marques da Silva et al. using 16S rRNA PCR and sequencing (31). DNA from none of the other RCB was detected. However, the DNA of periodontopathogens was detected in the atheromatous plaques from coronary arteries in a Chinese population with CP (46). The prevalence of *T. forsythia*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *P. gingivalis* was 31, 12, 18, and 33%, respectively. The same specific primers for *T. forsythia*, *F. nucleatum*, and *P. intermedia* were used by Cairo et al. (35) for investigating carotid atheromatous plaque for bacterial DNA. They were not able to detect DNA from these species, even though dental plaque samples were positive in 79, 63, and 53% of the cases, respectively.

Although we were able to detect bacterial DNA in most VD biopsies, the bacterial load was generally sparse as reflected by the amount of PCR products obtained. Also, with SEM several preparations from each biopsy had to be inspected in order to visualize bacterial cells. Most often the bacterial cells were found coaggregated in intravascular plaque lining the arterial lumen. Due to scant amounts of PCR products, several rounds of cloning and sequencing had to be performed to obtain 96 clone libraries with sequences of adequate quality and size, especially when processing VD biopsies from patients without periodontitis. In these patients, the final fraction of bacterial sequences averaged 25%, and the majority of the excluded sequences were background sequences and eukaryote sequences (Supplementary file SB). Thus, the number of vascular biopsies examined in this group was limited to 10 randomly selected biopsies. Renko et al. (47) using 16S rDNA sequencing, reported the average number of different bacterial sequences from atherosclerotic lesions in patients without signs of clinical infections to be 2.2 ± 1.2, which is comparable to our patients without CP (2.8 ± 1.6).

In subjects with CP, several common oral taxa were detected in the vascular biopsies, i.e. *Streptococcus*, *Prevotella*, *Capnocytophaga*, *Veillonella*, and *Porphyromonas* spp., while in patients without CP, only one common oral taxon was found (*Streptococcus* sp.). These species and other possible oral taxa detected in the vascular biopsies are highlighted in Tables 4 and 5. Although these taxa represent only a minor proportion of the oral bacterial microbiota, the data suggest that the oral cavity may be a potential source for bacterial dissemination to vascular tissue. Due to the near absence of RCB in vascular biopsies, we wanted to confirm their presence in the subgingival plaque in CP patients by using checkerboard DNA–DNA hybridization analysis. Although this method cannot be compared to 16S rDNA sequencing, it is an efficient and sensitive detection method for this purpose (30). The checkerboard analysis revealed presence of one to three members of the RCB in 7 out of 10 subgingival plaque samples from the CP patients (Table 2). Further, only 3 out of 19 bacterial spp. found in the subgingival plaque by checkerboard analysis were detected in the vascular biopsies in the CP group (i.e. *P. gingivalis*, *Actinomyces* sp., and *Streptococcus* spp.), suggesting dissimilar bacterial composition in the subgingival plaque and vascular biopsies. In a previous study, 16S rDNA-based PCR was positive for bacterial detection, whereas checkerboard DNA–DNA hybridization methods were negative. This indicated that the number of RCB-positive subgingival plaque samples in our study could possibly be even higher (48).

Similarly, Nakano et al. (49) showed that the bacterial composition in dental plaque was different from what was found in cardiovascular specimens. The presence of periodontopathogens and other oral bacterial species were sparse in cardiovascular tissue, and only a few species, including *S. mutans*, may have originated from the oral cavity.

A relatively high percentage (15.4%) of the sequences detected in vascular biopsies from patients with CP belonged to the *Enterobacteriaceae* family, i.e. *Serratia* sp. and *Klebsiella* sp. These and other enteric species have been isolated from subgingival sites (50). In addition, enteric bacteria are known to be important nosocomial pathogens, and it cannot be excluded that they were transmitted to the patients through the hospital environment (51). However, the highest load of *Enterobacteriaceae* is found as part of the gut microbiota. The intestines harbor the largest number of bacterial cells in the human body, making it likely that a major part of the bacterial taxa detected in our vascular biopsies had originated from the intestines. Several mechanisms for

dissemination of gut bacteria into the blood stream have been described (52). Koren et al. (45) found several operational taxonomic units shared between the gut and atherosclerotic plaque suggesting that bacteria present in the atherosclerotic plaque could have been derived from the gut. In a study by Hietbrink et al. (53), systemic inflammation induced by intravenously administrated *E. coli* lipopolysaccharide increased the intestinal permeability which may facilitate bacterial translocation. Animal studies have shown inflammation to induce and sustain increased intestinal permeability (54, 55). We may hypothesize that low-grade chronic systemic inflammation caused by periodontal disease (56) may affect intestinal permeability and thereby translocation of enteric bacteria, dissemination to the bloodstream, and further colonization of vascular lesions, as seen in our vascular biopsies from patients with CP.

We detected DNA from as-yet-uncultured bacterial taxa in both groups. Much of the human microbiota is not yet cultivated (57), and the presence of different not-yet-cultured bacterial taxa is to be expected. Their significance for oral and cardiovascular disease remains to be elucidated.

Several environmental bacterial species with undefined pathogenicity were detected in the VD biopsies, such as members of the families *Chitinophagaceae* and *Rhodobacteraceae*, *Geobacter* sp., and *Alishewanella* sp. Through the skin and mucous membrane linings of the respiratory, gastrointestinal, and genitourinary tracts, the human body is constantly exposed to environmental species. Some of the environmental bacteria detected in the VD biopsies may be part of our transient microbiota. The inclusion of negative controls and the high level of interindividual differences in detection of environmental species make it unlikely that these bacterial taxa are introduced by technical contamination. Several other studies have reported the presence of environmental species in vascular lesions and clinical samples (47, 58); however, the pathogenicity and clinical relevance of most environmental bacterial taxa in patient samples are yet to be determined.

Bacterial DNA belonging to *Pseudomonas* sp. and *Propionibacterium* acnes were frequently detected in both groups of patients. *Pseudomonas* is an opportunistic human pathogen of clinical relevance, and has been considered by some to be associated with periodontal disease (59). *P. acnes* is part of the commensal microbiota of skin and respiratory and digestive mucosa. This species is a common blood contaminant and can be the cause of postoperative infections, and has been associated with different cases of apical periodontitis and endocarditis (60, 61).

Acinetobacter sp. was found in both groups and was among the most prevalent in the group with CP. *Acinetobacter* has been isolated from periodontal pockets (50), and is known to be a frequent cause of nosocomial infections, especially pneumonias, as well as bloodstream and urinary tract infections (62, 63). *Streptococcus* and *Staphylococcus* spp. were detected in both groups. Non-pathogenic streptococcal spp. are part of the commensal microbiota of the mouth, skin, intestines, and upper respiratory tract. However, they can cause serious conditions such as bacteremia, meningitis, and pneumonia, and have been associated with endocarditis (64). *Staphylococcus* spp., such as *S. epidermidis* and *S. aureus*, is part of the commensal skin and respiratory tract microflora. Similar to streptococci they can cause cardiovascular conditions such as endocarditis (64).

By SEM, bacterial cells with different morphology were detected in all examined specimens from CP patients. Bacteria seemingly going through cell division were also observed. These findings emphasize the presence of bacterial cells, not only bacterial DNA, in the VD biopsies. Viable invasive bacteria such as *P. gingivalis* and *A. actinomycetemcomitans* have been cultivated from human atherosclerotic plaque (65, 66). Others have suggested that bacteria from the circulation might get caught in the vascular lesions as 'innocent bystanders' (67). However, animal studies have shown that bacterial taxa such as *Chlamydia pneumonia* and *P. gingivalis* may invade atherosclerotic plaque, alter inflammatory pathways, and cause molecular mimicry, and thereby may contribute to the progression of atherogenesis (6, 13).

In conclusion, our results indicate that the cumulative effect of different infectious agents may be associated with the pathophysiology of atherosclerotic and abdominal aortic aneurysmal vascular diseases. The infectious burden seems to be associated with chronic periodontal disease. However, periodontopathogens were rarely identified in the vascular biopsies, whilst gut bacteria were detected more frequently. A possible association between chronic periodontal disease, systemic inflammation, intestinal permeability, and enterobacterial colonization of atherosclerotic and aneurysmal tissue should be studied further.

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