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Prevalence of periodontal pathogens in subgingival lesions, atherosclerotic plaques and healthy blood vessels: a preliminary study

R. Elkaïm[1], M. Dahan[2], L. Kocgozlu[3], S. Werner[3], D. Kanter[2], J. G. Kretz[3,4], H. Tenenbaum[2,3]

[1]Parogène, Strasbourg, [2]Department of Periodontology, Dental Faculty, University Louis Pasteur, Strasbourg, [3]ERT 10-61, interne à l'Unité INSERM U 595, Strasbourg and [4]Department of Vascular Surgery, Medical Faculty, University Louis Pasteur, Strasbourg, France

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Background and Objective: Previous studies have reported different periodontal bacteria in atherosclerotic lesions, but their involvement in plaque formation remains unclear. The aim of the present study was to investigate the presence of 20 periodontal bacteria in atherosclerotic samples and healthy blood vessels (used as controls) and to clarify their relationship in regard to clinical and bacteriological periodontal status.

Material and Methods: The day before vascular surgery the patients had a thorough periodontal examination and bacteriological samples were taken from periodontally diseased sites. Atheromatous plaques, internal mammary arteries and saphenous veins were harvested during surgery. A DNA–DNA hybridization procedure was used to screen periodontal and vascular samples for the 20 selected bacterial species.

Results: Periodontal samples from the severe periodontitis group were found to have a higher prevalence and biomass of bacterial species than the moderate periodontitis group. In vessel samples, the prevalence of the same 20 bacterial species analyzed together was similar in the two groups, except for saphenous veins.

Conclusion: The presence of periodontal pathogens in atherosclerotic plaques and in apparently healthy vessels appeared to reflect a higher level of bacteremia rather than infection of endothelial cells.

Prof. Henri Tenenbaum, ERT 10-61, Faculté de Médecine, 11 rue Humann, Bâtiment 3, Etage 7–F-67085, Strasbourg cedex, France
Tel: +33 390243895
Fax: +33 390243842
e-mail: htenen@excite.com

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Epidemiological studies suggest that chronic oral infection, and especially periodontitis, may be an important risk factor for cardiovascular disease (1,2). Periodontal pathogens may enter the circulation during chewing, tooth brushing, flossing and gentle mastication (3,4). A causal link between periodontitis and cardiovascular disease has been postulated on the basis of the ability of some periodontal pathogens to invade arterial endothelial cells and smooth muscle cells, and thereby to promote platelet aggregation and thrombus formation (5). This local invasion can trigger a chronic inflammatory response that favours the onset and progression of atherosclerosis (6).

Chlamydia pneumoniae has been detected in atherosclerotic plaques (7) and periodontal pathogens have been detected in atherosclerotic vessels (8–12). Stelzel *et al.* (8) detected *Porphyromonas gingivalis*, but not

Aggregatibacter actinomycetemcomitans, in some aorta samples, whereas Marques da Silva *et al.* (9) found *A. actinomycetemcomitans*, but not *P. gingivalis*, in some aortic aneurysm samples. Kozarov *et al.* (11) detected viable periodontal bacteria (*P. gingivalis and A. actinomycetemcomitans*) in human atherosclerotic tissue and inferred that they might contribute to plaque instability and therefore to myocardial infarction or stroke. With the exception of Cairo *et al.* (10) and Padilla *et al.* (12), the studies mentioned above did not report the clinical or bacteriological periodontal status of the patients. Therefore, the detection of periodontal pathogens in vascular samples could not be directly correlated with the periodontal status of the patients. Moreover, the presence of periodontal pathogens within atherosclerotic lesions did not prove that these pathogens contributed in any way to the atherosclerotic process. Indeed, no studies have investigated the specificity of the presence of periodontal bacteria in atherosclerotic lesions compared with healthy blood vessels, such as saphenous veins or internal mammary arteries, used in cardiac artery bypass graft surgery.

Therefore, the aim of this study was to investigate the presence of periodontal pathogens in atherosclerotic and healthy blood vessels and to analyze their relationship with the clinical and bacteriological periodontal status.

Material and methods

Study population

The study was approved by the Ethics Committee of Strasbourg University Hospital and respected the Helsinki Declaration. All the participants gave their written informed consent to participate. Twenty-two patients undergoing cardiac artery bypass graft surgery at the Department of Vascular Surgery of Strasbourg University Hospital, France, were included in the study after meeting the following inclusion/exclusion criteria: at least 14 natural teeth; no scaling/root planing or systemic antibiotic therapy in the previous 6 mo; and no other systemic diseases (except for diabetes mellitus in three patients).

Periodontal assessment

Because our main inclusion criterion was for patients to undergo cardiac artery bypass surgery, which gave us the opportunity to analyze vascular specimens, periodontal examination could only be performed the day before vascular surgery. At six sites of all teeth, the same periodontist (M.D.) evaluated oral hygiene using the plaque index (13) and assessed gingival inflammation using the gingival index (14) and the sulcular bleeding index (15). Pocket depth was measured from the gingival margin to the most apical depth of the periodontal probe; recession was measured from the cemento–enamel junction to the gingival margin, and attachment loss was calculated by adding recession to pocket depth.

All the patients had periodontitis and were divided into two groups based on three criteria: mean pocket depth; mean attachment loss; and percentage of sites with pocket depth ≥4 mm. Eleven patients (nine men, two women; mean age 64.2 ± 9.1 years, range 46–77 years) with a mean pocket depth of < 3 mm, a mean attachment loss of < 4 mm and a percentage of sites with pocket depth ≥4 mm lower than 20% were included in the moderate generalized chronic periodontitis group and 11 patients (seven men, four women, mean age 55.1 ± 11.6 years, range 39–78 years) with a mean pocket depth of > 3 mm, a mean attachment loss of > 4 mm and a percentage of sites ≥4 mm higher than 20% were included in the severe generalized chronic periodontitis group. The clinical characteristics of the patients are shown in Table 1.

Bacteriological samples were collected from the four deepest periodontal pockets in each patient by inserting Mynol paper points into the pocket for 10 s, after drying the site and removing supragingival plaque. The paper points were immediately placed in Eppendorf tubes containing 0.5 mL of buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate) and processed for DNA extraction.

Vessel specimens

During surgery, performed the day after the periodontal examination, atheromatous plaques and specimens of internal mammary artery and saphenous vein grafts were harvested (11 specimens of each vessel type in each group). The specimens were 2–10 mm long and were placed in dry vials. All samples were immediately frozen in a bath of liquid nitrogen and stored at −80°C until DNA preparation.

DNA preparation from pocket samples

DNA was purified by protease treatment followed by phenol–chloroform–

Table 1. Clinical characteristics of patients with moderate generalized chronic periodontitis (mGCP) and severe generalized chronic periodontitis (sGCP)

Parameter	mGCP ($n = 11$)	sGCP ($n = 11$)	p-value
Gender (female:male)	2 : 9	4 : 7	NS
Age (years)	64.2 ± 9.1	55.1 ± 11.6	< 0.05
Current smoker	3 (27.3%)	1 (9.1%)	NS
Number of teeth	24.2 ± 3.3.	21.4 ± 4.0	NS
Plaque index	1.46 ± 0.57	1.69 ± 0.45	NS
Gingival index	1.33 ± 0.57	1.84 ± 0.64	NS
Sulcular bleeding index	0.77 ± 0.35	0.92 ± 0.71	NS
Probing depth	2.48 ± 0.27	3.43 ± 0.53	< 0.05
Recession	1.18 ± 0.61	1.44 ± 0.49	< 0.05
Attachment loss	3.40 ± 0.76	4.84 ± 0.85	< 0.05
Percentage of sites ≥4 mm	12 ± 6%	37 ± 15%	< 0.05

NS, nonsignificant; $p < 0.05$, significant difference between the mGCP group and the sGCP group (Student's t-test).
Values are mean ± standard deviation.

isoamylic alcohol extraction, as described previously (16). The DNA was precipitated by ethanol in the presence of 300 mM sodium acetate, pH 7, pelleted by centrifugation at 10,000 *g* for 15 min at 4°C, dried under vacuum and suspended in 1 mL of autoclaved 10 mM Tris-HCl (pH 7) containing 1 mM EDTA.

DNA preparation from tissue samples

Under a laminar flow hood, each tissue sample was slowly thawed into a sterile plastic tube containing 2 mL of sterile buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate). The samples were individually homogenized with a Polytron device (Kinematica, Luzern, Switzerland). RNAse A (10 μg/mL; Sigma, St Louis, MO, USA) was added to the homogenate for 1 h at 37°C and the homogenate was then treated with 50 μg/mL of proteinase K (Sigma) for 4 h at 55°C. DNA was extracted twice with phenol–chloroform–isoamylic alcohol and concentrated by adding 2 volumes of absolute ethanol in the presence of 300 mM sodium acetate, pH 7. DNA was pelleted by centrifugation at 10,000 *g* for 15 min at 4°C, then dried under a vacuum and suspended in 500 μL of autoclaved 10 mM Tris-HCl, pH 7, containing 1 mM EDTA. The DNA concentration was determined by measuring the absorbance at 260 nm, and purity was assessed from the 260:280 nm absorbance ratio.

Design of universal primers and probes for bacterial screening

The eubacterial primer set was designed from regions of identity within the 16S rDNA gene. We used a universal primer set with sequences 5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-GGTTACCTTGTTACGACTT-3′ (reverse) derived from previously published sequences (17). A specific probe was determined for each bacterial species from nonhomologous regions of the DNA amplicons obtained with the universal primer set. Specific probes were designed for 20 bacteria obtained from the American Type Culture Collection (Table 2). Each specific probe was checked for the absence of cross-hybridization with the 16S rDNA genes of all the other bacteria by using the BLAST program (18). As cross-hybridization could still occur between these 20 bacteria, we validated our primer set and probes with purified DNA prepared from each bacteria, with ranges from 0 to 10^9 bacteria/mL. No cross-hybridization was observed when purified DNA from each of the bacterial species was used up to the equivalent of 10^9 bacteria/mL. At higher doses ($> 10^9$ of a single bacterial species/mL or with $> 10^{10}$ of a bacterial mixture, equivalent to 5×10^8/mL for each of the 20 bacterial species), some cross-hybridizations appeared but with signals quantified only at level 1 (signal $< 10^3$ bacteria/mL) in our classification (see below in 'Bacterial screening'). This high dose was not detected in a clinical sample (i.e. a single species of bacteria is not present in amounts exceeding 10^8, representing 100% of the bacteria present.) and cross-hybridization, which would have changed the specific signal and the semiquantification, is therefore not possible. However, as more than 400 different bacterial species were found in periodontal pockets, we did not check all of them for cross-hybridization. Therefore, we cannot exclude the possibility that some bacterial species, not investigated in our study, could cross-hybridize with one or several of our specific probes. We also validated our primer set and selective

Table 2. The 20 bacterial species studied with sequences of specific probes

Bacteria	Probe sequence	ATCC no.
Aggregatibacter actinomycetemcomitans (*Aa*)	5′-GAGGATGGCCAGCCACACCG-3′	33384
Porphyromonas gingivalis (*Pg*)	5′-CGGGCGATACGAGTATTGCATTG-3′	33277
Prevotella intermedia (*Pi*)	5′-ACGTGGACCAAAGATTCATCGGTG-3′	25611
Campylobacter rectus (*Cr*)	5′-TTTCGGTGTAGGATGAGGCTATAT-3′	33238
Eikenella corrodens (*Ec*)	5′-GCGGGGGATCGCAAGACCTCGCGT-3′	23834
Tannerella forsythia (*Tf*)	5′-GCGATGGTAGCAATACCTGTCGGC-3′	43037
Actinomyces naeslundii (*An*)	5′-AGGGGCCTGCTTTTGTGGGTCCTG-3′	12104
Fusobacterium nucleatum (*Fn*)	5′-GAAAGCTATATGCGCTGTGAG-3′	10953
Prevotella nigrescens (*Pn*)	5′-GTGTTTCATTGACGGCATCCGATA-3′	33563
Streptococcus mutans (*Sm*)	5′-AACACACTGTGCTTGCACACCGTGTT-3′	25175
Streptococcus sanguinis (*Ss*)	5′-CTGAAGAGAGGAGCTTGCTCTTCTTGG-3′	29667
Streptococcus intermedius (*Si*)	5′-GTTTAAAAGGTGCAAATGCATC-3′	27335
Selenomonas noxia (*Sn*)	5′-ATGAAAGCTTGCTTTTATAAGCTT-3′	43541
Treponema denticola (*Td*)	5′-GCAAAGATCCGTCGGTAACGG-3′	35405
Veillonella parvula (*Vp*)	5′-CGATCTAACCTCGGCATCGAGGAA-3′	10790
Streptococcus oralis (*So*)	5′-AGAGTAGATGTTGCATGACATTTACTT-3′	35037
Capnocytophaga ochracea (*Co*)	5′- ATACAATCTGCCTTTCACTGGG-3′	27872
Porphyromonas endodontalis (*Pe*)	5′-GACGGAAAGATTTATTGATTACAG-3′	35406
Prevotella melaninogenica (*Pm*)	5′-AGTCTTCGATGACGGCATCAG-3′	25845
Eubacterium nodatum (*En*)	5′-AGCAGAGCATTCGCATGGATGA-3′	33099

A eubacterial universal primer set was used to amplify a region of identity within the 16S rDNA gene of the 20 bacteria species. A bacterial specific probe was designed from nonhomologous regions of the DNA amplicons obtained with the universal primer set. ATCC no, American Type Culture Collection number.

probes for possible cross-hybridization with bacteria from nonoral sources, such as the gut. No cross-hybridization was detected with purified human DNA and with DNA purified from three different strains of *Escherichia coli* (up to the equivalent of 10^9 bacteria/mL). Primers and probes were synthesized and purified by Eurobio (Les Ullis, France). All the bacteria were grown at 37°C under anaerobic conditions in the media recommended in the American Type Culture Collection technical bulletin. Bacterial density was determined by examining several dilutions in a hemocytometer.

Polymerase chain reaction

Polymerase chain reaction (PCR) amplification of the 16sDNA gene was performed by using 100 ng of tissue DNA or 1 μL of subgingival DNA solution, the universal primer set (0.2 μM each), in a total volume of 25 μL, under the conditions recommended by the *Taq* DNA polymerase manufacturer (Euromedex, Strasbourg, France). After an initial denaturation step at 95°C for 3 min, the PCR reaction consisted of 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 5 min.

Each set of experiments included two negative controls, one consisting of sterile distilled water and the other of 10 ng/mL of salmon sperm DNA instead of template DNA. Positive controls contained purified DNA from a mixture of all the test species (a final concentration of 10^7 bacteria/mL, composed of 5×10^5 of each bacterium/mL). Serial dilutions of this positive-control DNA (from 10^7 to 10^2 total bacteria/mL) served to estimate the quantity of a bacterial species.

Bacterial screening

We used a modified checkerboard DNA–DNA hybridization procedure (19). Each PCR product was denatured and rapidly loaded into one of the 30 lanes of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA) containing a Hybond-N membrane (Amersham, Aylesbury, UK). Adsorbed DNA was covalently fixed to the membrane by baking at 80°C for 2 h. As each of the 20 specific probes required a different temperature for either the hybridization step or the high-stringency posthybridization washing steps, it was not possible to use the whole membrane on the Miniblotter 25 (Immunetics). Consequently, 30 strips of 0.5 cm width (one for each bacterial species) were cut from the membrane. Each strip was prehybridized for 6 h at 56°C and then hybridized overnight with 15 ng/mL of a 3′ digoxygenin-labelled specific probe, as previously described (20). The strips were washed twice for 15 min in ×5 sodium chloride/sodium citrate buffer containing 0.1% sodium dodecyl sulfate at room temperature and then for 30 min in ×0.2 sodium chloride/sodium citrate buffer containing 0.1% sodium dodecyl sulfate at a higher temperature, depending on the probe melting temperature (generally melting temperature was −5°C). Hybrids were then revealed on each strip by a chemiluminescence reaction with an antibody to digoxigenin conjugated to alkaline phosphatase, as previously described (21). The strips were then exposed to autoradiography film and the band intensity obtained for each sample was quantified on the film by comparison with a standard curve obtained from serial dilutions of the positive control DNA, as follows: class 0, no signal; class 1, $< 10^3$ bacteria/mL; class 2, $> 10^3$ bacteria/mL and $< 10^5$ bacteria/mL; class 3, $> 10^5$ bacteria/mL. These classes were not considered in vessel samples.

Statistical analysis

All clinical and microbiological measurements were averaged per patient, so the unit of measurement was the patient. Differences in clinical parameters between the moderate and severe periodontitis groups were analyzed using the Student's *t*-test.

Differences in percentages of bacterial DNA detected in periodontal sites were analyzed using Fisher's exact test.

The differences in the number of positive patients for a given bacterium detected in each vessel type were analyzed using Fisher's exact test. Statistical comparisons were also performed between the number of positive patients in each bacterial class. Differences were considered significant when $p < 0.05$. All statistical tests were performed with SIGMASTAT version 2.0 software (Jandel Corporation, Erkrath, Germany). No adjustments for multiple testing were carried out because of the explorative nature of this study.

Results

Periodontal samples

Sixteen of the 20 bacterial species were more prevalent in subgingival plaque samples from the severe periodontitis group than from the moderate periodontitis group (Fig. 1). Moreover, bacterial classes 1–3 in these subgingival plaque samples were significantly higher in the severe periodontitis group than in the moderate periodontitis group (Fig. 2). *P. gingivalis and Fusobacterium nucleatum* were among the bacteria detected at higher levels (class 3) in the two groups of patients (data not shown).

Vessel samples

Comparisons between the two groups of patients did not show significant differences in the prevalence of any of the 20 bacteria in mammary arteries, whereas significant differences appeared for 9 of the 20 bacteria in saphenous veins and only for *Campylobacter rectus* and *Eikenella corrodens* in atheromatous plaques (Table 3). In atheromatous plaques, only one of the test species bacteria, *E. corrodens*, was more prevalent in the moderate generalized chronic periodontitis group, but the differences between groups was not statistically significant.

In combined vessel samples, 15 of 20 bacteria were more prevalent in the severe generalized chronic periodontitis group than in the moderate generalized chronic periodontitis group, but statistically significant differences were observed for only 8 of these 15 bacteria

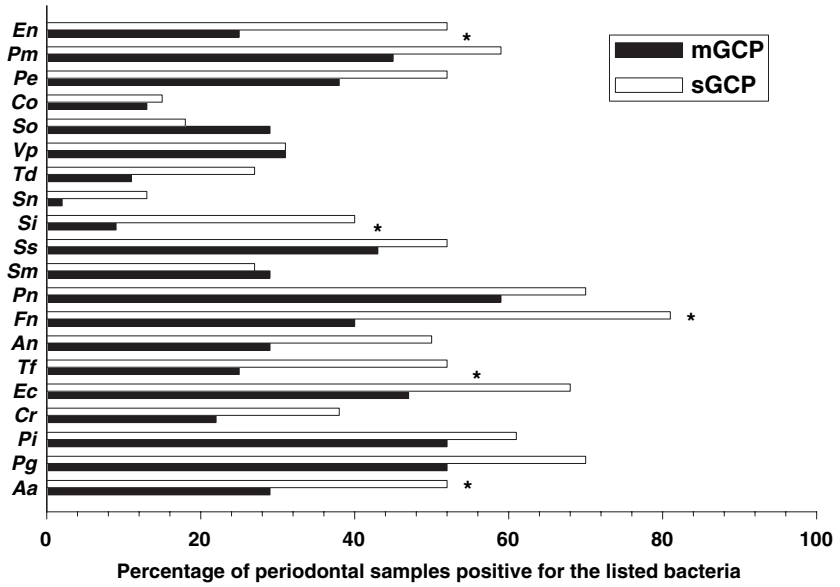

Fig. 1. Percentages of samples positive for the listed periodontal bacteria. Bacteriological samples were collected and pooled from the four deepest periodontal pockets of 11 patients with moderate generalized chronic periodontitis (mGCP) and of 11 patients with severe generalized chronic periodontitis (sGCP). *Statistically significant difference. *Aa*, *Aggregatibacter actinomycetemcomitans*; *An*, *Actinomyces naeslundii*; *Co*, *Capnocytophaga ochracea*; *Cr*, *Campylobacter rectus*; *Ec*, *Eikenella corrodens*; *En*, *Eubacterium nodatum*; *Fn*, *Fusobacterium nucleatum*; *Pe*, *Porphyromonas endodontalis*; *Pg*, *Porphyromonas gingivalis*; *Pi*, *Prevotella intermedia*; *Pm*, *Prevotella melaninogenica*; *Pn*, *Prevotella nigrescens*; *Si*, *Streptococcus intermedius*; *Sm*, *Streptococcus mutans*; *Sn*, *Selenomonas noxia*; *So*, *Streptococcus oralis*; *Ss*, *Streptococcus sanguinis*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*; *Vp*, *Veillonella parvula*.

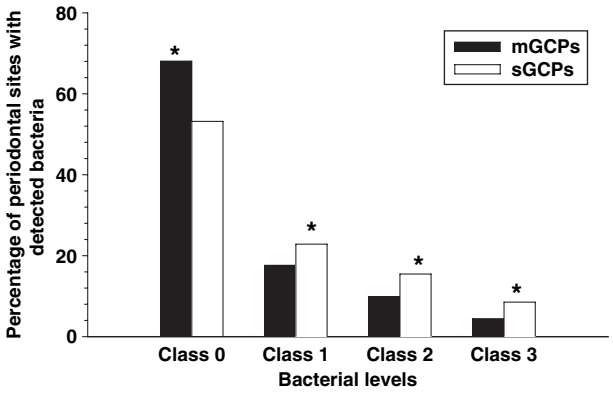

Fig. 2. Mean prevalence of the three bacterial classes in subgingival plaque samples. The mean prevalence of the three bacterial classes in subgingival plaque samples was taken from 11 patients with moderate generalized chronic periodontitis and from 11 patients with severe generalized chronic periodontitis. A total of 44 periodontal sites were analyzed per group (i.e. four diseased sites were analyzed per patient). *Statistically significant difference.

(Table 3), including the pathogens *P. gingivalis and F. nucleatum*.

Although the number of different bacterial species found in each vessel type was higher in the severe generalized chronic periodontitis group than in the moderate generalized chronic periodontitis group, a significant difference was observed only for saphenous vein samples (Table 4).

Discussion

Some case-control and cohort studies have shown significant associations between oral infection and cardiovascular diseases, with odds ratios ranging from 1.2 to 2.7 (22).

Different hypotheses have emerged to link oral infections, especially periodontal diseases, with the development and progression of atherosclerosis: direct invasion of the vascular endothelium by pathogens; trafficking of pathogens; immunological sounding; and stimulation of an auto-immune response via molecular mimicry. One or a combination of these mechanisms may contribute to pathogen-accelerated atherosclerosis (23).

Screening of human atheroma has revealed that several pathogens, including *C. pneumoniae and P. gingivalis*, are frequently detected in atherosclerotic samples (24,25). In a recent study by Kaklikkaya *et al.* (26), *C. pneumoniae* was detected in atherosclerotic plaques, but not in healthy vessel samples. None of the studies referring to periodontal pathogens detected in atherosclerotic plaques, except those of Cairo *et al.* (10) and Padilla *et al.* (12), checked the periodontal status of the patients. Therefore, it was difficult to conclude that pathogens found in atheromatous plaques were first located in periodontal pockets of the affected patients. Moreover, no previous study has investigated the presence of periodontal bacteria simultaneously in atherosclerotic plaques and in healthy, control vessels, such as saphenous veins or internal mammary arteries. Such a comparison should highlight the specific presence of periodontal bacteria in atherosclerotic lesions, which could strengthen the hypothesis for a direct role of these pathogens in the atherosclerotic process.

In the current investigation, we used PCR to analyze the specific periodontal microbiota in subgingival sites, atheromatous plaques and healthy blood vessels from patients undergoing coronary artery bypass grafting. Although our selected primer set and probes have given us the best sensitivity/specificity of detection, cross-reactions with bacteria from nonoral sources, such as the gut, or

Table 3. Number of patients with statistically significant differences between the two groups for the listed bacterial species in saphenous vein, atheromatous plaque and combined vessel samples

	Saphenous veins			Atheromatous plaques			Combined vessel samples		
Bacteria	mGCP ($n = 11$)	cGCP ($n = 11$)	p-value	mGCP ($n = 11$)	cGCP ($n = 11$)	p-value	mGCP ($n = 11$)	cGCP ($n = 11$)	p-value
Aggregatibacter actinomycetemcomitans	0	6	< 0.05						
Porphyromonas gingivalis	0	6	< 0.05				5	11	< 0.05
Prevotella intermedia	0	6	< 0.05				3	11	< 0.05
Campylobacter rectus	0	6	< 0.05	0	6	< 0.05	3	11	< 0.05
Eikenella corrodens				5	0	< 0.05			
Fusobacterium nucleatum	0	6	< 0.05				3	11	< 0.05
Streptococcus sanguinis	0	6	< 0.05				2	11	< 0.05
Streptococcus oralis	0	6	< 0.05				5	11	< 0.05
Prevotella melaninogenica	0	6	< 0.05				3	11	< 0.05
Eubacterium nodatum	0	6	< 0.05				0	11	< 0.05

For mammary arteries, no significant difference was observed between the two groups of patients for any of the 20 bacterial species (not shown).
mGCP, moderate generalized chronic periodontitis; sGCP, severe generalized chronic periodontitis.

Table 4. Number of different bacterial species detected in mammary artery, saphenous vein and atheromatous plaque samples

	Mammary arteries			Saphenous veins			Atheromatous plaques		
	mGCP	sGCP	p-value	mGCP	sGCP	p-value	mGCP	sGCP	p-value
Bacteria	11	18	NS	0	18	< 0.05	9	12	NS

mGCP, moderate generalized chronic periodontitis; NS, not significant; sGCP, severe generalized chronic periodontitis.

with currently unknown periodontal bacteria could not be excluded.

In the blood vessels, we found that periodontal pathogens were more prevalent in the severe generalized chronic periodontitis group than in the moderate generalized chronic periodontitis group, but a significant difference was observed only for saphenous vein samples. Indeed, our results favour the hypothesis that periodontal pathogens detected at a higher prevalence in diseased periodontal sites are also detected at a higher prevalence in vascular samples. However, unlike *C. pneumoniae*, which has been detected in atherosclerotic plaques but not in healthy vessels, periodontal pathogens were detected in both diseased and healthy samples. According to the severity of the periodontal disease, these bacteria enter the circulation more easily and can subsequently be detected in blood vessels and atherosclerotic lesions. Therefore, bacterial presence appeared to reflect a higher level of bacteremia rather than infection of endothelial cells. The chronic and cyclic nature of periodontal diseases provides multiple opportunities for repeated dissemination of pathogens in the blood.

On the one hand, periodontal pathogens could increase the incidence and severity of transient bacteremia through gingival ulceration and vascular changes in the periodontal tissues. On the other hand, bacteremia could affect endothelial integrity, metabolism of plasma lipoproteins, blood coagulation and platelet function (23).

Human studies suggest that shortly after dental treatment *P. gingivalis* bacteremia is evident (27). *P. gingivalis*-specific DNA and rRNA have been detected in carotid atheromatous plaques obtained from periodontally affected patients (24). These studies indicated that *P. gingivalis* present in periodontal pockets gained access to the vasculature and that either a bacteremia or a bacteremia followed by invasion of the vessel endothelium was responsible for the presence of this virulent microorganism in these plaques (23).

Although the magnitude of the systemic microbial stress induced by chronic periodontal diseases may be small compared with severe acute infections, such as pneumonia, periodontitis is both widespread and often persists for decades. Furthermore, moderate forms of periodontitis affect 75% of the population, whereas severe forms affect $\approx 15\%$ of the population, indicating that the total burden of this infection may be highly significant. The pocket epithelium surface area in patients with periodontitis may be as large as 8–20 square centimetres (28). The loss of epithelial integrity within the pathological, periodontal pockets creates the opportunity for bacteria to disseminate because the total number of subgingival bacteria in periodontitis individuals could be estimated at 10^7–10^8 (29). Recently, it has been suggested that patients with a history of coronary heart disease show a considerably higher total periodontal pathogen burden in their subgingival biofilm than age- and gender-matched controls without a history of coronary heart disease (3). Moreover, the same study has shown a higher number of *A. actinomycetemcomitans*, but not of

P. gingivalis, in the subgingival biofilm of patients with coronary heart disease compared with controls.

In our study, we showed that the subgingival bacterial prevalence and bacterial biomass were higher in patients with severe periodontitis. Furthermore, the prevalence of periodontal pathogens detected in all the vessel samples was also higher in the severe periodontitis group. These data suggest that the higher the abundance of periodontal pathogens in the subgingival microbiota, the greater the number of bacteria that are likely to enter the circulation, with possible involvement of some bacteria (i.e. *P. gingivalis*) in the pathogenesis of atheromatous plaques. *P. gingivalis* adhesion to and invasion of epithelial cells through major fimbriae (FimA) and cysteine proteinases (gingipains) has been demonstrated (30). *P. gingivalis* can also adhere to and infect cultures of coronary endothelial cells (31), leading to the activation and expression of adhesion factors (32). *A. actinomycetemcomitans* probably invades endothelial cells via a mechanism dependent upon the engagement of the platelet-activating factor receptor by bacterial phosphorylcholine (33).

However, as healthy internal mammary artery and saphenous vein specimens showed a higher prevalence of periodontal bacteria than atheromatous plaques in our group of patients with severe periodontitis, the role of a direct invasion of vascular endothelium by pathogens in the development and progression of atherosclerosis still remains questionable. It is conceivable that, upon entering the vasculature, which is an immunologically privileged site, the host mounts a response to the periodontal pathogens while they are circulating in the vessels, independent of pathogen–endothelium interactions. Thus, the immunological response to blood-borne infection may be sufficient to stimulate the host immunologically in a way that promotes atherosclerosis.

Recent data have suggested that chronic periodontal infections could create an environment in some vessels that is conducive to atheroma development by the secretion of soluble mediators from periodontal pockets into the circulation. There are components of periodontitis and atherosclerosis that are similar in terms of pathophysiology, and some risk indicators have been shown to be elevated in both pathologies. Recent data implicates inflammation, and possibly autoimmunity, in the pathogenesis of atherosclerosis (34). Inflammation may initiate or contribute to the progression of the atherosclerotic plaque, which, in many ways, is similar to what happens in the diseased periodontal tissues. The activation of vascular endothelium in response to inflammatory cytokines would shift the balance of the vascular endothelium from a 'healthy' normal, antithrombotic environment to a 'diseased' prothrombotic environment (35). Amar *et al.* (36) reported that severe periodontal disease was associated with endothelial dysfunction and systemic inflammation in relatively young patients compared with carefully matched healthy controls. Khovidhunkit *et al.* (37) suggested that periodontitis may affect the metabolism of lipoproteins, a major contributor to atherosclerosis. Elevated C-reactive protein levels, another contributor to the pathogenesis of atherosclerosis, have been found in patients with periodontitis (38). Craig *et al.* (39) showed that patients with chronic destructive periodontitis exhibited 100% higher levels of C-reactive protein in their serum than those with mild, less destructive periodontitis. Patients affected with both atherosclerosis and periodontitis possess antibodies that react with *P. gingivalis* heat shock proteins (24). Thus, locally produced pro-inflammatory mediators are likely to find their way into the systemic circulation and may affect distant organs, including the cardiovascular system.

The focus of future studies will be to clarify the precise molecular mechanisms by which periodontal pathogens could contribute to the progression of atherosclerosis. Indeed, infection of human endothelial cells with *P. gingivalis* and activation by its lipopolysaccharide induce changes in the expression of some cellular components, such as heat-shock protein 60, known to be involved in the severity of the atherosclerotic process (40).

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References

1. Madianos PN, Bobetsis GA, Kinane DF. Is periodontitis associated with an increase risk of coronary heart disease and preterm and/or low birth weight births? *J Clin Periodontol* 2002;**29:**22–36.
2. Scannapieco FA, Bush RB, Paju S. Associations between periodontal disease and risk for atherosclerosis, cardiovascular disease and stroke. A systematic review. *Ann Periodontol* 2003;**8:**38–53.
3. Spahr A, Klein E, Khuseyinova N *et al.* Periodontal infections and coronary heart disease. *Arch Intern Med* 2006;**166:**554–559.
4. Forner L, Larsen T, Kilian M, Holmstrup P. Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. *J Clin Periodontol* 2006;**33:**401–407.
5. Pussinen PJ, Mattila K. Periodontal infections and atherosclerosis: mere associations? *Curr Opin Lipidol* 2004;**15:**583–588.
6. Dorn BR, Burks JN, Seifert KN, Progulske-Fox A. Invasion of endothelial and epithelial cells by strains of *Porphyromonas gingivalis*. *FEMS Microbiol Lett* 2000;**187:**139–144.
7. Maass M, Bartels C, Engel PM, Mamat U, Sievers HH. Endovascular presence of viable *Chlamydia pneumoniae* from a carotid endorterectomy specimen. *J Infect Dis* 1998;**176:**292–295.
8. Stelzel M, Conrads G, Pankuweit S *et al.* Detection of *Porphyromonas gingivalis* DNA in aortic tissue by PCR. *J Periodontol* 2002;**73:**868–870.
9. Marques da Silva RDA, Caugant PS, Lingaas O, Geiran L, Tronstad Olsen I. Detection of *Actinobacillus actinomycetemcomitans* but not bacteria of the red complex in aortic aneurysms by multiplex polymerase chain reaction. *J Periodontol* 2005;**76:**590–594.
10. Cairo F, Gaeto C, Dorigo W *et al.* Periodontal pathogens in atheromatous plaques. A controlled clinical and laboratory trial. *J Periodont Res* 2004;**39:**442–446.
11. Kozarov EV, Dorn BR, Shelburne CE, Dunn WA Jr, Progulske-Fox A. Human atherosclerotic plaque contains viable invasive *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*.

Arterioscler Thromb Vasc Biol 2005;**25:**17–18.

12. Padilla C, Lobos O, Hubert E *et al*. Periodontal pathogens in atheromatous plaques isolated from patients with chronic periodontitis. *J Periodont Res* 2006;**41:** 350–353.
13. Silness J, Loe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. *Acta Odontol Scand* 1964;**22:**121–135.
14. Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* 1963;**21:**533–551.
15. Muhleman HR, Son S. Gingival sulcus bleeding – a leading symptom in initial gingivitis. *Helv Odont Acta* 1971;**15:**107–113.
16. Dix K, Watanabe SM, McArdle S *et al*. Species-specific oligodeoxynucleotide probes for the identification of periodontal bacteria. *J Clin Microbiol* 1990;**28:**319–323.
17. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. *Nucleic Acid Techniques in Bacterial Systematics*. Wiley: Chichester, 1990:115–175.
18. Altschul SF, Gish W, Miller W, Myers E, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;**5:**403–410.
19. Socransky SS, Smith C, Martin L *et al*. ''Checkerboard'' DNA–DNA hybridization. *Biotechniques* 1994;**17:**788–792.
20. Tenenbaum H, Elkaim R, Cuisinier F, Dahan M, Zamanian P, Lang JM. Prevalence of six periodontal pathogens detected by DNA probe method in HIV *vs* non-HIV periodontitis. *Oral Dis* 1997;**3:** 153–155.
21. Bolcato-Bellemin AL, Elkaim R, Tenenbaum H. Expression of RNAs encoding for alpha and beta integrin subunits in periodontitis and in cyclosporin A gingival overgrowth. *J Clin Periodontol* 2003;**30:** 937–943.
22. Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S. Periodontal disease and cardiovascular disease. *J Periodontol* 1996; **67:**1123–1137.
23. Geerts SO, Legrand V, Charpentier J, Albert A, Rompen EH. Further evidence of the association between periodontal conditions and coronary artery disease. *J Periodontol* 2004;**75:**1274–1280.
24. Gibson FC 3rd, Yumoto H, Takahashi Y, Chou HH, Genco CA. Innate immune signaling and *Porphyromonas gingivalis*-accelerated atherosclerosis. *J Dent Res* 2006;**85:**106–121.
25. Hu H, Pierce GN, Zhong G. The atherogenic effects of Chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae*. *J Clin Invest* 1999; **103:**747–753.
26. Kaklikkaya I, Kaklikkaya N, Buruk K *et al*. Investigation of *Chlamydia pneumoniae* DNA, chlamydial lipopolysaccharide antigens, and *Helicobacter pylori* DNA in atherosclerotic plaques of patients with aortoiliac occlusive disease. *Cardiovasc Pathol* 2006;**15:**105–109.
27. Cavrini F, Sambri V, Moter A *et al*. Molecular detection of *Treponema denticola* and *Porphyromonas gingivalis* in carotid and aortic atheromatous plaques by FISH: report of two cases. *J Med Microbiol* 2005;**54:**93–96.
28. Hujoel PP, White BA, Garcia RI, Listgarten MA. The dentogingival epithelial surface area revisited. *J Periodont Res* 2001;**36:**48–55.
29. Loos BG. Systemic markers of inflammation in periodontitis. *J Periodontol* 2005; **76:**2106–2115.
30. Chen T, Nakayama K, Belliveau L, Duncan MJ. *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. *Infect Immun* 2001;**69:**3048–3056.
31. Deshpande RG, Khan MB, Genco CA. Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. *Infect Immun* 1998;**66:**5337–5343.
32. Khlgatian M, Nassar H, Chou HH, Gibson FC 3rd, Genco RJ. Fimbria-dependent activation of cell adhesion molecule expression in *Porphyromonas gingivalis*-infected endothelial cells. *Infect Immun* 2002;**70:**257–267.
33. Schenkein HA, Barbour SE, Berry CR, Kipps B, Tew JG. Invasion of human vascular endothelial cells by *Actinobacillus actinomycetemcomitans* via the receptor for platelet-activating factor. *Infect Immun* 2000;**68:**5416–5419.
34. Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999;**340:**115–126.
35. Vita JA, Loscalzo J. Shouldering the risk factor burden: infection, atherosclerosis and the vascular endothelium. *Circulation* 2002;**106:**164–166.
36. Amar S, Crokce N, Morgan S, Loukideli M, Van Dyke TE, Vita JA. Periodontal disease is associated with brachial artery endothelial dysfunction and systemic inflammation. *Arterioscler Thromb Vasc Biol* 2003;**23:**1245–1249.
37. Khovidhunkit W, Memon RA, Feingold KR, Gunfeld C. Infection and inflammation-induced proatherogenic changes of lipoproteins. *J Infect Dis* 2000;**181:**462–472.
38. Czerniuk MR, Gorska R, Filipiak KJ, Opolski G. C-reactive protein in patients with coexistent periodontal disease and acute coronary syndromes. *J Clin Periodontol* 2006;**33:**415–420.
39. Craig RG, Yip JK, So MK, Boylan RJ, Socransky SS, Haffajee AD. Relationship of destructive periodontal disease to the acute-phase response. *J Periodontol* 2003; **74:**1007–1016.
40. Chung SW, Kang HS, Park HR, Kim SJ, Kim SJ, Choi JI. Immune responses to heat shock protein in *Porphyromonas gingivalis*-infected periodontitis and atherosclerosis patients. *J Periodont Res* 2003;**38:** 388–393.