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Original Article

The presence of periopathogenic bacteria in subgingival and atherosclerotic plaques– An age related comparative analysis

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Abstract

Introduction: There is a known connection between periodontitis and atherosclerosis and the presence of periopathogens in blood vessels. However, changes of the oral microflora related to the aging process and its possible effects on atherosclerosis, have yet to be analyzed. The aim of this study was to assess temporal changes in the frequency of periodontal bacteria in the subgingival plaque and in atherosclerotic blood vessels of patients with atherosclerosis.

Methodology:The study included 100 patients with atherosclerosis and periodontitis, divided into two groups, below and over 60 years of age. Clinical examinations were performedand subgingival plaque specimens were collected as well as biopsy specimens from the following arteries: coronary (34), carotid (29), abdominal (10), femoral (10), mammary (13) and iliac (4). Subgingival and artery specimens were subjected to PCR detection of 5 major periodontal pathogens: *Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Aggregatibacter actinomycetemcomitans (Aa), Tannerella forsythensis (Tf) and Treponema denticola (Td*).

Results:*Tf* was the most and *Td* the least frequent bacteria in both age groups and in both types of samples. The frequencies of bacteria in subgingival versus atherosclerotic samples were: *Tf* (76%:53%), *Pi* (71%:31%), *Pg* (60%:38%), *Aa* (39%:14%) and *Td* (21%:6%). Only *Aa* and *Pi* showed a significant difference of prevalence between younger and older patients. The most colonized artery was a. coronaria, followed by a. carotis, a. abdominalis, a. mammaria, and a. femoralis.

Conclusions: Patient's age and the distance of a given blood vessel from the oral cavity influenced microbiological findings in the atherotic plaque.

Key words: periodontitis; atherosclerosis; periodontal microorganisms.

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Introduction

Both periodontitis and atherosclerosis are common diseases in human adults. Periodontitis involves the destruction of supporting structures of the teeth including periodontal ligament, bone and soft tissues, and causes tooth loss [1,2]. The disease is a chronic low grade infection caused by bacteria that populate periodontal pockets. The microbes involved in periodontal disease are largely Gram-negative anaerobic bacilli with some anaerobic cocci and anaerobic spirochetes [3].

Atherosclerosis (AS) has been defined as a progressive disease that causes focal thickening of walls of arteries. In the past years there has been an emerging body of evidence that infectious processes may contribute to atherosclerosis [4-6]. Reports of association between a variety of infectious agents and atherosclerosis have appeared [7-11]. Herpes simplex

virus (HSV) has been proposed in the 1970s as a possible causative agent in the development of atherosclerosis in experimental animals [9]. Another member of the herpesviridae family, cytomegalovirus (CMV), the Gram-negative bacteria, *Chlamydia pneumoniae* and *Helicobacter pylori* have been also added to the list of possible infective agents [11].

The link between dental infections and cardiovascular diseases began with epidemiological associations [12-15]. Randomized controlled clinical trials, longitudinal, cohorts, and case-control studies have also suggest an association between periodontal disease (PD) and atherosclerosis [16]. Studies in which periodontal and atherosclerotic status were assessed with more accuracy, have shown a clear association between atherosclerosis and periodontal status as measured by odds ratios [17-19].

A number of comprehensive publications have demonstrated the presence of periodontal pathogens in atherosclerotic plaques in human atheroma samples [10,20,21] and connections between periodontitis and atherosclerosis have also been established in animal models [22-26] demonstrating that the increase of atheromatous lesions was associated with induced exposure to periodontal pathogenic bacteria. At present a clear relationship has been established between the degree of periodontal destruction and the presence of periopathogens.

However, one aspect of the relationship between atherosclerosis and oral pathogenic microorganisms has been relatively neglected - the temporal change of oral microflora and its possible effects on AS. Namely, it has been documented that oral microflora undergoes substantial changes along with the aging process, and periodontal pathogens have been found even on dental prostheses of elderly edentulous patients [27,28].

This study was designed in order to estimate a possible change in the bacterial DNA presence in the subgingival biofilm and atherosclerotic blood vessels in relation to patients' age. The second goal was to estimate weather the distance of the artery from the oral cavity had any influence on its colonization. Oral and arteries samples have been analyzed for the presence of five major periodontal pathogens (*Porphyromonas gingivalis, Prevotella intermedia, Agreggatibacter actinomycetemcomitans, Tannerella forsythensis* and *Treponema denticola*).

Methodology

Patients and sampling

One hundred specimens of subgingival plaque of patients with moderate to severe periodontal disease and 100 specimens of their respective atherosclerotic blood vessels obtained during vascular surgery procedures, were subjected to periodontal bacteria detection. A positive diagnosis of atherosclerosis was based on clinical findings, coronary angiography and Doppler echosonography performed at the Center for Vascular and Endovascular Surgery at the Clinical Center of Serbia and the Institute for Cardiovascular Diseases of the Clinical Center "Dr Dragiša Mišović" Dedinje, Belgrade. Samples of atherosclerotic plaques belonged to the following six groups in relation to the localization of atherosclerosis: carotid arteries (29), abdominal aortic aneurysm (10), femoral arteries (10), illiac arteries (4), coronary arteries (34), mammary arteries (13). Basic clinical data obtained through clinical chartings and interviews are given in Fig 1. There were 69 male and 31 female patients, aged 28 to 94 years, mean 59.2. The patients were divided into 2 age groups (A) 28-59 and (B) 60 to 94. Patients that had been taking antibiotics in the previous three months and/or who had received periodontal treatment in the last 6 months were excluded from the study. Oral samples were collected after periodontal examination. The tooth was isolated by sterile cotton rolls, and supragingival plaque was removed using sterile gauze and curette. Two sterile paper points size 30 were placed until mild resistance into the pocket for 30 seconds. Subgingival and atherosclerotic samples were placed in a vial, immediately frozen and stored at -20°C.

The local Ethical Committee reviewed and approved the study protocol. All the patients signed a written informed consent prior to all the procedures. The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008).

Sample preparation

Bacterial DNA from atherosclerotic plaques of blood vessels was isolated by treating the samples with 10% aqueous solution of proteinase K (Thermo Fisher Scientific, Waltham, MA, USA) at 56 °C for 30 minutes, followed by inactivation of the enzyme by heating the samples at 94 °C in a thermoshaker (Biosan TS-C100, Riga, Latvia) for 15 minutes. In order to isolate bacterial DNA from subgingival plaque samples, paper points were submersed in 300 µL of 50 mM NaOH solution, then vortexed for 10 seconds and heated at 95 °C for 5 minutes. Then, thirty microliters of Tris-HCl (pH =8) solution was added to previous mixture and samples were centrifuged at 12100 × g at room temperature for 2 minutes. Finally, supernatant

Table 1. Primers used for PCR, their hybridization temperatures and product sizes.

Species	Sequence (5'-3')	Hybridization temperature (°C)	Product size (bp)
Tannerella forsythensis	Fwd GCGTATGTAACCTGCCCGCA Rv TGCTTCAGTGTCAGTTATACCT	55	600
Porphyromonas gingivalis	Fwd AGGCAGCTTGCCATACTGCG Rv ACTGTTAGCAACTACCGATGT	55	400
Prevotella intermedia	Fwd CGTGGACCAAAGATTCATCGGTGGA Rv CCGCTTTACTCCCCAACAAA	55	259
Aggregatibacter actinomycetemcomitans	Fwd GCTAATACCGCGTAGAGTCGG Rv ATTTCACACCTCACTTAAAGGT	55	500
Treponema denticola	Fwd TAATACCGAATGTGCTCATTTACAT Rv TCAAAGAAGCATTCCCTCTTCTTCTTA	60	316

containing bacterial DNA was transferred into sterile plastic tubes. All samples were stored at -20 °C prior to PCR analysis.

Microorganisms detection

The presence of 16S ribosomal DNA of *Porphyromonas gingivalis*, *Tannarella forsythensis*, *Prevotella intermedia*, *Treponema denticola* and *Actinobacillus actinomycetemcomitans* was detected using the standard PCR method. Positive controls were strains obtained from American Type Culture Collection (ATCC): *A. actinomycetemcomitans* (ATCC 33384), *P. gingivalis* (ATCC 33277), *P. intermedia* (ATCC 33563), *T. forsythensis* (ATCC 43037) and *T. denticola* (ATCC 35405). For negative controls PCR reactions were performed with water instead of bacterial DNA. Twenty five microliters of aqueous mixture containing: 2.5 µl of PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of species specific primers, 1 U of DreamTaq DNA polymerase (all products from Thermo Fisher Scientific™; Waltham, MA, USA) and 5 µL of bacterial DNA isolate, were used for the reaction. PCR was performed in a thermal cycler (Peqlab PeqSTAR 2X; Erlangen, Germany) under the following conditions: initial denaturation (95 °C for 3 minutes), cycling (35 rounds of: denaturation (94 °C for 45 seconds), hybridization (appropriate temperature for each pair of primers for 60 seconds), and elongation (72 °C for 60 seconds)), and final elongation (72 °C for 5 minutes). PCR products were separated by polyacrylamide gel electrophoresis, stained with ethidium bromide and finally visualized and photographed after exposure to UV light. All primer sequences and hybridization temperatures are given in Table 1.

Statistical analysis

The methods of descriptive statistics, such as measures of central tendencies, were used to describe the results of this study. The Chi square test was carried out to compare basic epidemiological and clinical characteristics, as well as the prevalence of periopathogenic bacteria between examined groups. A p-value lower than 0.05 was considered statistically significant. All statistical analyses were performed on Statistical Package for the Social Sciences software, version 22.0 (IBM SPSS Inc., Chicago, IL, USA).

Results

As expected, the two age groups of patients with periodontal disease and atherosclerosis showed a statistically significant difference in regard to the incidence of hypertension, diabetes and family history of cardiovascular diseases (Figure 1). Other diseases such as malignancies, endocrine and hematological disorders etc. were rare and did not differ between the groups (data not shown).

As much as 91% of oral samples were positive for at least one of the tested microorganism, while in the group of atherosclerostic arteries there were 69% of positive samples. The decreasing frequencies of microorganisms in oral specimens versus artery specimens were: *Tf*. (76%:53%), *Pi* (71%:31%), *Pg* (60%:38%), *Aa* (39%:14%) and *Td* (21%:6%) and are given in Figure 2.

Figure 1. Basic epidemiological and clinical characteristics of the two age groups of patients.

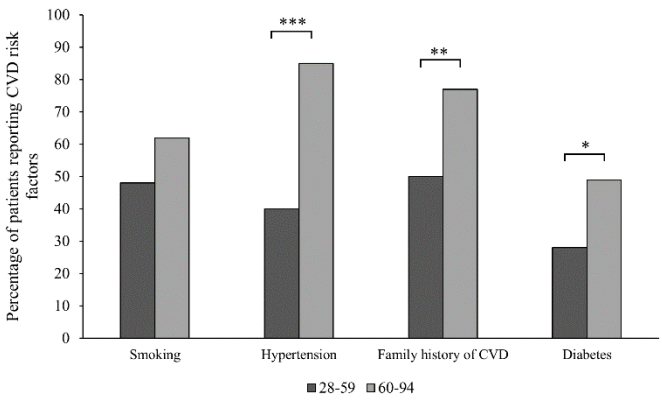
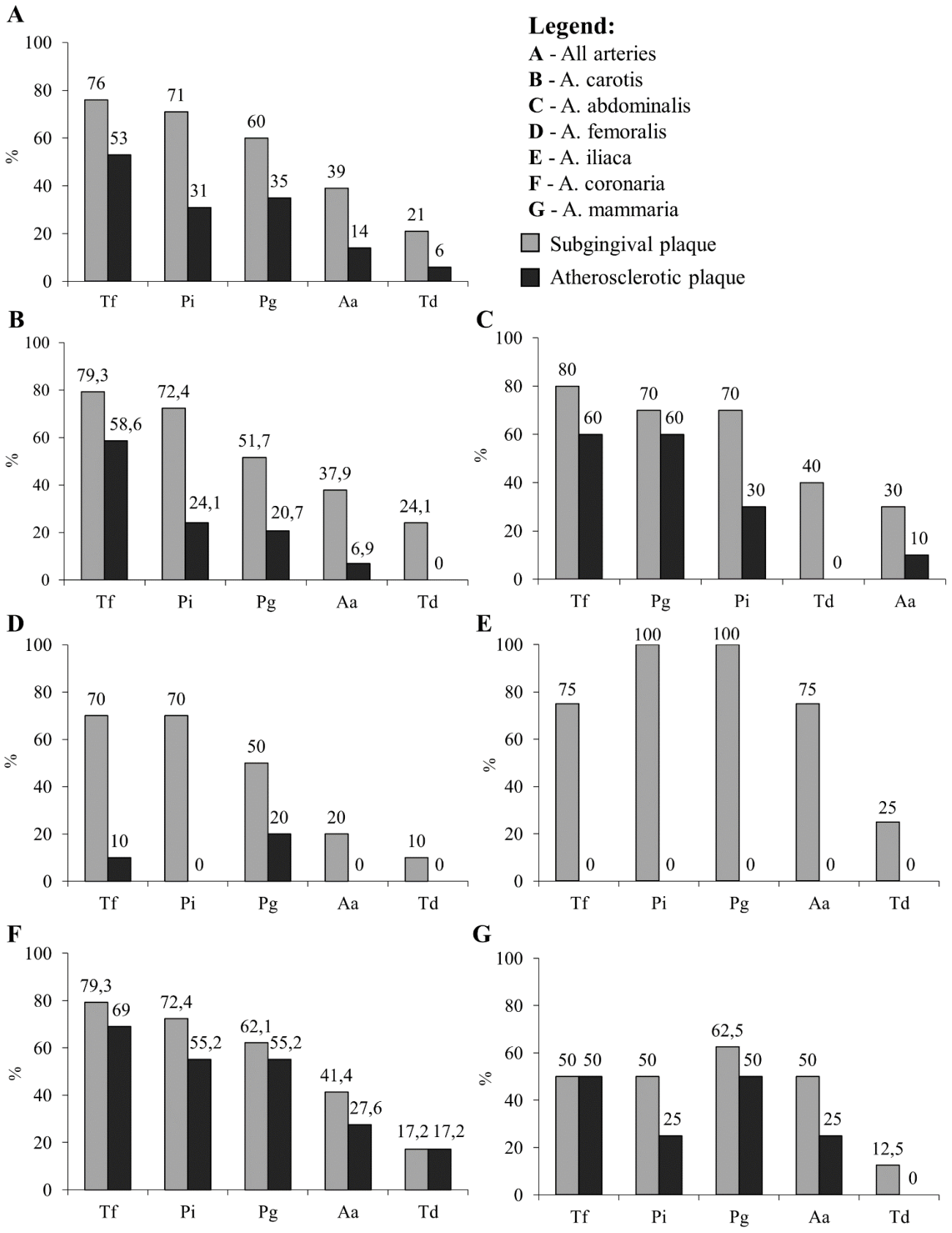


Figure 2. Comparison of microorganisms' incidence in subgingival versus atherosclerotic plaques in the entire sample, regardless of patients' age.



The ratio of oral versus artery bacteria presence was as follows: *Tf*:1.4, *Pg*:1.6, *Pi*:2.3, *Aa*:2.8 and *Td*:3.5. It seems that *Tf* had the highest propensity to colonize arteries and *Td* the lowest.

Coronary arteries were the blood vessels in which microorganisms were most frequently found, followed by carotid, abdominal and mammary arteries. Oral pathogens were rare in femoral and could not be detected at all in iliac arteries (Figure 3).

When focusing on bacterial prevalence changes in artery samples, in relation to the process of aging, statistically significant difference between younger and older patients were found for *Pi* and *Aa* (Figure 4). Differences between younger and older patients were also noted when considering the number of different bacterial species per blood vessel (Figure 5).

Discussion

The frequency of detection of periodontal bacteria using PCR methods in various atherosclerotically altered blood vessels differ among studies published in the past two decades. In our study *T. forsythensis* was detected as the most prevalent microorganism, both in subgingival and atherosclerotic plaques, without significant changes related to patients' age. The second most prevalent microorganism in oral samples but not in artery samples was *P. intermedia*. We detected *T. forsythensis* in 76%, *P. intermedia* in 71%, *P. gingivalis* in 60%, *A. actinomycetemcomitans* in 39% *T. denticola* in 21% of subgingival plaque samples. The same bacteria were present in atherosclerotic artery walls at lower percentages and with a slightly changed order: *T. forsythensis* in 53%, *P. gingivalis* in 38%, *P. intermedia* in 31%, *A. actinomycetemcomitans* in 14% and *T. denticola* in 6% of samples.

P. intermedia serves as a primary colonizer of oral keratinocytes and a fundament for secondary colonizers, such as *P. gingivalis*. *P. intermedia* is designated as a potent periodontal pathogen responsible for aggressive forms of periodontitis, acute necrotizing ulcerative gingivitis and some severe systemic infections [10,19].

P. gingivalis is one of the most important causative agents of periodontal disease [29]. Thanks to the plethora of virulence factors this periopathogenic microorganism skips the surveillance of the hosts defense mechanisms and induces target tissue destruction [30,31].

Although considered as important periodontal pathogens, *A. actinomycetemcomitans* and *T. denticola* were less represented in the studied sample.

Figure 3. The magnitude of blood vessels colonization by different oral pathogenic microorganisms (the y axis represents the total number of cases with a given bacteria).

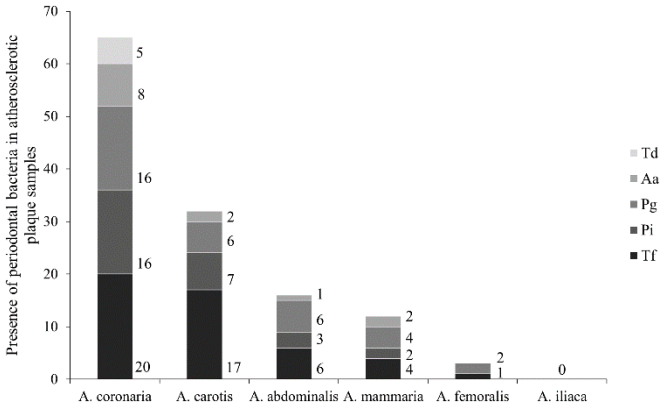


Figure 4. Microorganism prevalence in arteries of younger and older patients with AS.

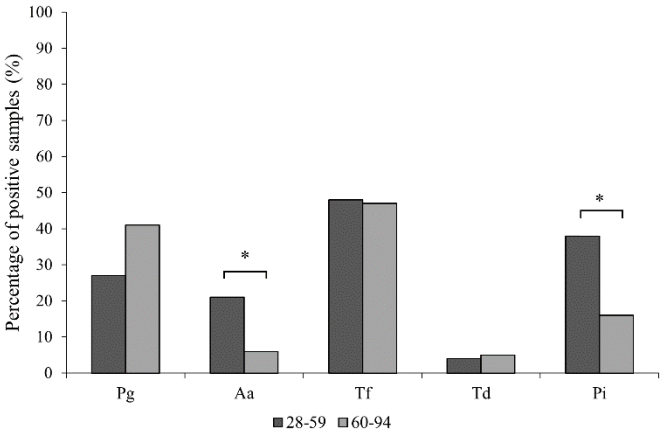
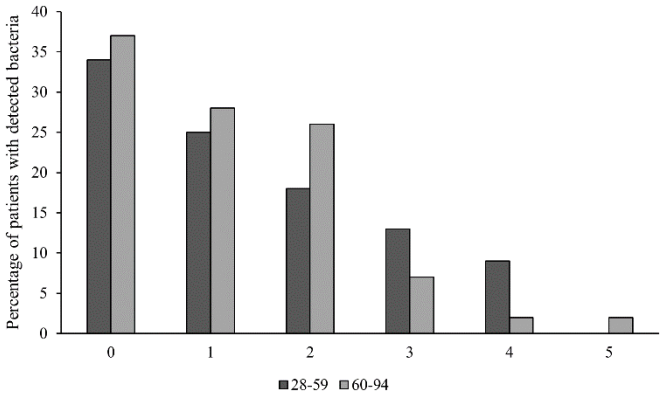


Figure 5. Percentage of artery samples with 0, 1, 2, 3, 4 or 5 different bacterial species in relation to patients' age group.



Interestingly, only the incidence of *P. gingivalis* increased in arteries with patients' age. *T. denticola* and *T. forsythensis* incidence remained unchanged while *A. actinomycetemcomitans* and *P.intermedia* showed a statistically significant incidence drop in the older group of patients. A similar drop for *A. actinomycetemcomitans* with aging has been previously described [28].

Harazsthy at al. reported that 44% of 50 atheromas were positive for at least 1 of targeted periodontal pathogens [32]. Stelzel at al. found only four out of 26 aortic tissue samples clearly positive for P. gingivalis while *A. actinomycetemcomitans* was not detected [33]. Interestingly, Cairo at al. detected *T. forsythensis* in 79%, *F. nucleatum* in 63%, *P. intermedia* in 53%, *P. gingivalis* in 37% and *A. actinomycetemcomitans* in 5% subgingival plaque samples of patients scheduled for carotid endarterectomy, but no periodontal bacteria DNA in any of the carotid artery samples [21]. Mahendra et al. found more bacterial presence in subgingival plaque samples compared to atherosclerotic plaques of coronary arteries, but opposite to our results, *T. denticola* was the most detected pathogen (66.7% positive oral specimens vs. 51% positive artery specimens). The least detected bacteria in their study was *A. actinomycetemcomitans* that was found only in 2% of subgingival plaque samples [34]. Another group of Indian researchers found similar distribution of periopathogens in the same oral and arterial samples with exception of *P. intermedia* which showed higher prevalence in coronaries [9]. They found no *A. actinomycetemcomitans* presence neither in subgingival nor in atherosclerotic samples [9]. In contrast to these and to our study, Figuero et al. detected *A. actinomycetemcomitans* at higher rates in oral samples (72.2%), but only in 3.0% of blood vessel samples [35]. The present study, which covers six types of atherosclerotic vessels, showed a high percentage of positive samples for one or more periodontal bacteria. These findings represent a clear evidence that periodontal pathogens enter the circulation, causing transient bacteraemia, and then can lodge in diseased blood vessel walls. The closer the vessel to oral cavity, the higher the chances that microorganisms will colonize it. In other words, there is a bacterial "gradient" which reflects the distance of a given artery from the primary bacterial source, the oral cavity. The distribution of microorganisms in the subgingival plaque was affected by aging, and this was also reflected in their distribution in arteries. In our study, we could not detect bacteria in iliac arteries. The small number of specimens and the distance from the oral cavity may explain the negative result.

One of the major shortcomings of this and other reports dealing with the relationship between periodontal disease and atherosclerosis [10,12,16,17] is that clinical measurements represent only one point in time. If periodontal inflammation and exposure to periodontal gram negative bacteria do play a role in the pathogenesis of atherosclerosis the effect would most likely be seen with long term exposure of the blood stream to bacteremia and inflammatory mediators coming from the periodontium. Moreover, the extent of exposure of the arteries to circulating agents originating in the periodontal tissues is hard to define when there are missing teeth. If all the lost teeth were periodontally healthy and extracted because of caries or trauma, then edentulous subjects would be expected to have minimal exposure to periodontal gram negative bacteria and cytokines. However, if teeth were lost due to periodontal disease the risks of arterial invasion by bacteria and cytokine derived from periodontal tissues, would be dependent on the time these infected and inflamed regions were present prior to extraction. This latter scenario is more likely as statistical connections between missing teeth and manifestations of arterial involvement are generally found [13,17].

Still, in order to more plainly investigate the role of periodontal pathology on atherosclerosis, precise measurements of exposure times and intensity of exposure to periodontal disease would be necessary, i.e. more complex and specific measurements of periodontal and plaque status in a larger time window should be developed.

Conclusions

Oral pathogens were found at high frequency in the walls of atherosclerotic blood vessels and reflected their incidence in the subgingival plaque, although with a slightly lower frequency and change in the order (Tf>Pi>Pg>Aa>Td versus Tf>Pg>Pi>Aa>Td). Patient's age and the distance of a given blood vessel from the oral cavity influenced microbiological findings.

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