Microbiological, periodontal and blood biochemistry profile of periodontal patients with atherosclerosis.
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Abstract
Aims: This study aims (i) to describe the periodontal health status and blood biochemistry profile of patients with atherosclerosis, and (ii) to examine periodontal pockets and atheroma for simultaneous presence of five periodontal pathogens. Methods: 31 patients scheduled for carotid endarterectomy were enrolled in this study. A complete periodontal and clinical examination was conducted before surgery. Subgingival samples and atheromatous plaques were microbiologically analyzed with the polymerase chain reaction technique, and atheromatous plaques were histologically analyzed. Results: Subgingival and atheroma samples tested positive to at least one of five target periodontopathogens. P. gingivalis, T. forsythia and F. nucleatum showed a strong link between the concomitant presence of these bacteria in atheroma and periodontal pockets (48.39%, 38.71% and 51.61% of patients, respectively). The absence of P. intermedia and A. actinomycetemcomitans in both locations was demonstrated in 74.19% and 93.54% of patients, respectively. Increased serum glucose and triglyceride levels (> 6.1 mmol/L and >1.7 mmol/L, respectively) were correlated with the presence of T. forsythia, F. nucleatum and P. intermedia (pvalue <0.1) in atheroma samples. Increased lymphocytes and gingival bleeding index values indicated a closely association with the presence of the target periodontopathogens in atheroma (pvalue <0.1). Conclusion: The presence of P. gingivalis, F. nucleatum, and T. forsythia in subgingival plaque suggests that these bacteria contribute to the development of atherosclerosis. Alterations in some biochemical parameters might promote bacterial colonization and metabolism, and therefore contribute to the development of atherosclerosis.

Keywords
Atherosclerosis, cardiovascular diseases, chronic periodontitis, oral biofilm, Molecular diagnosis.

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**Introduction**

Chronic periodontitis is a widespread inflammatory oral disease (1,2) caused by the subgingival accumulation of predominantly Gram-negative anaerobic bacteria (3). The pathogenesis of this disease, characterized by chronic destruction of the dental support tissues, may lead to tooth loss if periodontitis is left untreated (4).

Several studies (5-8) have suggested that chronic periodontitis may be a risk factor in the development of certain systemic diseases, such as cardiovascular diseases (9-15), respiratory diseases (16), diabetes mellitus (17), preterm low-weight births (18,19), hyperlipidemia (20,21), and osteoporosis (22). Some hypotheses have been put forward to support these associations, but the high number of common risk factors behind periodontitis and atherosclerosis, and the ethical limitations to design studies to obtain a high level of scientific evidence, make it difficult to ascertain the real impact of one disease over the other. Some research claims that the association between periodontitis and atherosclerosis may arise from a focal infection that triggers a chronic inflammation leading to an atherosclerotic process, as well as the spread of periodontal bacteria into the bloodstream through the gingiva, inducing continuous benign bacteremia that may lead to the development of atherosclerosis (23-30).

Some studies on patients undergoing endarterectomy have detected periodontopathogens in atheroma plaques (31-63) but few (31-44) have evaluated their simultaneous presence in the atheroma and periodontal pocket, although these studies do not appear to have taken into account the common risk factors in the development of both diseases. Differences in sample numbers, medical and periodontal data collection, and bacterial detection methods may have resulted in conflicting findings. The different study designs suggest the need for a consensus to optimize bacterial detection methods and conduct a reproducible protocol to standardize this type of study (64). Tables S1 and S2 list the studies published in this field and summarize their methodological characteristics.

The information published in the scientific literature demonstrates the link between periodontitis and atherosclerosis, however there is insufficient information about the periodontal health status and the blood biochemistry profile in patients suffering both diseases. For this reason, this study aimed (i) to describe the periodontal health status and blood biochemistry profile of patients with atherosclerosis, and (ii) to examine periodontal pockets and atheroma for simultaneous presence of five periodontal pathogens.

**Methods**

*Patients and specimens*

Thirty-one adult patients with carotid stenosis requiring endarterectomy were selected for this study. A day before surgery, a completed medical history was taken with a questionnaire in order to obtain information about each patient’s identification, age, and medical and familiar histories. All patients underwent a complete blood count, blood chemistry analysis, coagulation test, measurement of acute-phase reactants (C-reactive protein and fibrinogen), and assessment of the risk factors for atherosclerosis and/or periodontal disease, which included hypertension and hyperlipidemia, diabetes mellitus and smoking.

None of the patients had received any antibiotic therapy in the previous 3 months. Patients with additional chronic
infections and/or edentulism patients were excluded.

The study protocol was authorized by the Ethics Committee of the Red Cross General Hospital (Cardiovascular Unit, Barcelona, Spain) and conducted in accordance with the Helsinki Declaration. All patients received oral and written information about the characteristics of the study and were given written informed consent before taking part in the study.

Periodontal examination

Preoperatively, all patients included were examined for periodontal disease by a periodontal specialist. Oral hygiene habits (frequency of toothbrush, interproximal hygiene and use of mouthwash) were registered, and patients were submitted to a complete periodontal exploration of plaque index (full-mouth plaque core), bleeding on probing (full-mouth bleeding score), probing depth (PD) and clinical attachment level (CAL). The periodontal diagnosis was performed in accordance with the American Association of Periodontology Guidelines (65), and patients were classified into four groups: periodontally healthy (<1 mm CAL), mild chronic periodontitis (1–2 mm CAL), moderate chronic periodontitis (3–4 mm CAL), and severe chronic periodontitis (>5 mm CAL).

Periodontal and atheroma sample collection

After performing periodontal diagnosis, supragingival plaque was removed with sterile curettes and subgingival samples were obtained by inserting a sterile paper point for 10s into the deepest periodontal pockets of each quadrant (39). The paper points were placed in a sterile test tube containing a buffer solution (1x Tris-EDTA buffer pH 7.5, 10 min, 95°C), quantified and tested with human X chromosome specific-primers. Approximately 175 ng of genomic DNA was added to the standard PCR mixture in a 50µl final volume. Final reactions contained 5µL of 10x buffer solution without MgCl2 (GeneCraft®), 2.5µL 50mM MgCl2 (GeneCraft®), 5µL 2.5mM dNTP’s (GeneCraft®), 0.5µL 5µ/µL Taq polymerase (GeneCraft®), 0.5µL 10µM of each primer, and 1µL of template DNA. A sequence of nearly 20 bp of the 16 rRNA gene of five periodontopathogens (A. actinomycetemcomitans, P. gingivalis, F. nucleatum, T. forsythia and P. intermedia) was amplified by using specific primers described by Ashimoto et al. (66). Table 3 shows primer specifications. The temperature profiles included an initial denaturation step at 95°C for 1 min and 94°C for 30 s, followed by 30 cycles of 61°C (A. actinomycetemcomitans, T. forsythia) or 64°C (P. intermedia, P. gingivalis, F. nucleatum) for 2 min (annealing), 72°C for 40 s (extension), and a final extension of 72 °C for 1 min. Positive (DNA from pure bacterial culture) and negative controls (only reagents without DNA) were included in each PCR reaction. Electrophoresis was performed with a 2% agarose gel.
Table 1. Specific primers for target periodontal bacterial DNA amplification

<table>
<thead>
<tr>
<th>Periodontal bacteria</th>
<th>Ref (*)</th>
<th>Primer pairs (5' → 3')</th>
<th>Position and Molecular Weight (bp)</th>
</tr>
</thead>
</table>
| A. actinomycetemcomitans | a | F: atgccaaacttgacgttaaat  
R: aaaccccatctcgagttctttcc | 478 – 1034 (557) |
|                       | b | F: gtgcgaaagcgtggggagca  
R: cttcgggtatgctcaagagtagg | 769 – 998 (230) |
| P. gingivalis          | a | F: aggcagcttgccataactgcg  
R: ctgttaacaaactacgagtcg | 729 – 1132 (405) |
|                       | b | F: cgggttatcacaacagctatcgaat  
R: gggtgcccgtcgttatgcaaatc | 732 – 1075 (344) |
| P. intermedia         | a | F: cgtggaccaagattcatcg  
R: atacgttgcgtgcactca | 172 – 638 (575) |
|                       | b | F: ggttaaaggtgcgtctatt  
R: agttccctcggtcctgctc | 196 – 257 (62) |
| F. nucleatum          | a | F: gatgaacgtgacgatagaatgc  
R: tcatcggtcacaagatagttgtgc | 27 – 400 (392) |
|                       | b | F: acgggtgagtaacgcgttacaag  
R: cccctttggtgcgccgatccccc | 89 – 364 (276) |
| T. forsythia          | b | F: tacaggggaataaaatgagatacg  
R: acgtcatcccaacctcttcctc | 404 – 1149 (746) |

(*): “a” Ashimoto et al. (66); “b” this study; F: forward primer; R: reverse primer
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Carotid atheroma bacterial identification

A modified phenol-chloroform method was performed to extract and purify DNA from the atheroma specimens (67). Nested-PCR was used to enhance the threshold detection level because of the suspected low bacterial load in atheroma plaques. The first PCR run to identify subgingival bacteria is described above. For the second PCR run, 10 µl of the first PCR DNA product was added to a 50 µl final volume. Internal specific primer sequences of nearly 20 bp were obtained from the NCBI designing tool (Table 1). Primers were previously validated with positive controls, and detection limits of PCR system were established at 10^1/ml. The annealing temperature was first calculated with the NetPrimer software and then experimentally determined for each target bacterium. Amplification was performed for 30 cycles with an annealing temperature of 50°C (T. forsythia) or 55°C (A. actinomycetemcomitans, P. gingivalis, F. nucleatum and P. intermedia). Positive and negative controls were included in each PCR reaction.

Histological analysis of atheroma plaques

The upper sections of atheroma samples were immersed in paraffin and stained with hematoxylin and eosin for histological analysis. Lymphocytes and PMNs neutrophils cells/field were counted (magnification x400). Inflammatory pattern was classified into three different categories, depending on the number of inflammatory cells/field (68). Inflammation was considered “acute” when >50% of the inflammatory cells were PMN neutrophils, “subacute” in the presence of 50% PMN neutrophils and 50% lymphocytes, and “chronic” when lymphocytes were predominant (>50%). Three different fields of each histological preparation were analyzed.

Statistical analysis

Measurements were statistically evaluated using a commercially available software program (Statgraphics Plus, version 5.1 for Windows, MD, USA). Descriptive analysis was used to analyze medical and periodontal characteristics in terms of percentage (%). Prevalence of periodontal pathogens detected in atheroma and periodontal pockets, as well as the simultaneous presence in both locations was calculated with frequency analysis. Tendencies of relation between clinical and microbiological features were assessed using multifactorial ANOVA analysis (pvalue <0.1).

Results

Periodontal and general medical health status and blood biochemistry profile

Table 2 shows the most important clinical and periodontal features from the study population. A total of 11 women and 20 men between 51 and 83 years old (mean age: 73±6.61) with a low socioeconomic status, and a sedentary lifestyle took part in this study. The mean Body Mass Index (BMI) was higher in women than in men (mean: 30.42 and 29.09, respectively) and most of the patients suffered hypertension and diabetes mellitus (86.2% women; 79.3% men). The mean Bleeding Index of the population was 60.33. Twenty percent (20%) of patients were periodontally healthy or presented gingivitis. Mild chronic periodontitis was diagnosed in 16% of the population, and more severe forms were diagnosed in the rest of the population (12% moderate and 52% severe). Biochemistry analyses revealed levels of triglycerides, HLD cholesterol, leucocytes and Srm-Glucose above expected healthy values in the study population.
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Table 2. Clinical and periodontal characteristics of the study population.

<table>
<thead>
<tr>
<th>General medical data</th>
<th>Mean &amp; SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>73 ±6.61</td>
</tr>
<tr>
<td>Females (%)</td>
<td>31.03</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>74.32 ±11.09</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>28.80 ±3.42</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>156.06 ±19.5</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>80.32 ±12.44</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>74.19</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Smokers and ex-smokers (%)</td>
<td>67.75</td>
</tr>
<tr>
<td>Smokers &gt;10 years (%)</td>
<td>51.61</td>
</tr>
<tr>
<td>Smokers &gt; 10 cigarettes/day (%)</td>
<td>51.61</td>
</tr>
<tr>
<td>Periodontal examination</td>
<td></td>
</tr>
<tr>
<td>Mean Bleeding Index (gingivitis)</td>
<td>60.33 ±24.36</td>
</tr>
<tr>
<td>Toothbrush at least once/day (%)</td>
<td>80.5% ±0.9</td>
</tr>
<tr>
<td>Interproximal hygiene (%)</td>
<td>9.67 ±0.3</td>
</tr>
<tr>
<td>Use of mouthwash (%)</td>
<td>35.48 ±0.48</td>
</tr>
<tr>
<td>Biochemistry</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (0.45-1.70 mmol/L)*</td>
<td>2.06 ±1.28</td>
</tr>
<tr>
<td>Cholesterol (≤ 5.2 mmol/L)*</td>
<td>5.38 ±1.12</td>
</tr>
<tr>
<td>HDL Cholesterol (&gt; 0.90 mmol/L)*</td>
<td>1.17 ±0.25</td>
</tr>
<tr>
<td>LDL Cholesterol (&lt; 3.40 mmol/L)*</td>
<td>2.91 ±0.61</td>
</tr>
<tr>
<td>Fibrinogen g/L (2.00-5.00)*</td>
<td>2.99 ±0.44</td>
</tr>
<tr>
<td>Leucocytes (4.00-11.00 x10⁹/L)*</td>
<td>8.86 ±2.69</td>
</tr>
<tr>
<td>Neutrophils (2.50-7.50x10⁹/L)*</td>
<td>5.75 ±2.63</td>
</tr>
<tr>
<td>Lymphocytes (1.50-4.50x10⁹/L)*</td>
<td>2.16 ±0.66</td>
</tr>
<tr>
<td>Monocytes (0.2-0.8x10⁹/L)*</td>
<td>0.69 ±0.27</td>
</tr>
<tr>
<td>Eosinophiles (0.05-0.5x10⁹/L)*</td>
<td>0.21 ±0.17</td>
</tr>
<tr>
<td>Basophiles (&lt;0.15x10⁹/L)*</td>
<td>0.03 ±0.018</td>
</tr>
<tr>
<td>Srm Glucose (3.9-6.1 mmol/L)*</td>
<td>7.23 ±2.17</td>
</tr>
<tr>
<td>Triglycerides (0.45-1.70 mmol/L)*</td>
<td>2.06 ±1.28</td>
</tr>
</tbody>
</table>

* range values in health, SD Standard Deviation
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Histological characteristics of atheromatous plaque

Table 5 shows the characteristics of the study population. Histological analysis of atheroma samples revealed that all plaques presented crystallized cholesterol deposits, and that the subacute inflammation pattern was the most prevalent (54.83%). Acute and chronic inflammations were less frequent (16.35% and 28.82%, respectively). Figure 1a-c shows an example of each local inflammatory pattern.

Table 5. Histological characteristics of the study population.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Mean &amp; SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atheroma plaque length (mm)</td>
<td>15.41 ±4.45</td>
</tr>
<tr>
<td>(upper sections)</td>
<td></td>
</tr>
<tr>
<td>Atheroma diameter (mm)</td>
<td>7.77 ±2.77</td>
</tr>
<tr>
<td>Inflammatory area (mm²)</td>
<td>1.99 ±2.01</td>
</tr>
<tr>
<td>Subacute inflammatory pattern (%)</td>
<td>54.83</td>
</tr>
<tr>
<td>Chronic inflammatory pattern (%)</td>
<td>28.8</td>
</tr>
</tbody>
</table>

* range values in health, SD Standard Deviation

Microbiological analysis of atheroma and periodontal samples

All subgingival and atheroma samples tested positive to at least one of the five target periodontal pathogens. The analysis of subgingival plaque samples showed that F. nucleatum was the most frequent bacteria (90.32%), followed by T. forsythia (3.87%), and P. gingivalis (64.51%). A. actinomycetemcomitans and P. intermedia were less frequently detected (6.45% and 3.22%, respectively) (Figure 2).

Figure 1a-c. Histological images of atheromatous plaques with optical microscope (x400).

This figure shows an example of the three local inflammatory patterns found in the histological analysis. Fig. 1a Inflammatory pattern, where PMN neutrophils (PMN) are the most commonly observed cells in the study area (> 50%). Fig. 1b Subacute inflammatory pattern, where PMN neutrophils (PMN) and lymphocytes (L) increase at a similar rate. Fig. 1c Chronic inflammatory pattern, where lymphocytes are most frequently observed in the study area (> 50%).
Atheroma samples showed a high presence of A. actinomycetemcomitans (80.64%), P. gingivalis (70.96%), T. forsythia (51.61%) and F. nucleatum (61.29%). P. intermedia was detected in a lower percentage of atheromas (22.58%) (Figure 3).
Figure 4 shows the frequency of simultaneous detection of periodontopathogens in the atheroma and periodontal pockets. F. nucleatum, P. gingivalis, and T. forsythia presented a high simultaneous detection in both locations (periodontal pocket and atheroma plaque of the same patient). F. nucleatum was detected in 16 out of 19 patients (51.61%), P. gingivalis in 15 out of 22 patients (48.39%), and T. forsythia in 12 out of 16 (38.71%). The absence of P. intermedia and A. actinomycetemcomitans in both locations was determined in 74.19% and 19.34% of the patients, respectively.

Multifactorial analysis recorded a significant correlation between the detection of T. forsythia and F. nucleatum in carotid atheroma samples and the colonization of these microorganisms in subgingival sites (p-value <0.1). Although the frequency of simultaneous detection for P. gingivalis was higher than the frequency of other bacteria, the correlation analysis was not statistically significant. Additionally, increased serum glucose and triglycerides levels (> 6.1 mmol/L and >1.7 mmol/L, respectively) were correlated with the presence of P.intermedia (p-value <0.1), T. forsythia and F. nucleatum in atheroma samples. Increased lymphocytes and BI values were closely linked with the presence of the five target periodontopathogens in atheroma (p-value <0.1) (data not shown).
Discussion

Several studies have demonstrated that periodontitis might increase the risk of some cardiovascular diseases (10,15,69-72). However, the level of association remains controversial, because both diseases share common risk factors (i.e. smoking, diabetes mellitus, BMI, lifestyle, sex, age, etc.) that might act as “confounding factors” and complicate the determination of the level of association.

For ethical reasons, it is difficult to design studies that aim to determine the level of association between both diseases. Taking this limitation into account, the present study aimed to evaluate the periodontal health status and “blood biochemistry profile of atherosclerotic patients, and to ascertain the presence of periodontal bacterial DNA in subgingival plaque samples and carotid atheromas.

As demonstrated in previous studies, the PCR technique, given its high detection sensitivity range, offers a valid molecular method for detecting periodontal bacteria. However, some authors using the conventional PCR system have been unable to detect periodontal bacterial DNA in atheroma samples with this molecular diagnostic method (31,36,38). These negative findings could be explained by the low bacterial load contained in the atheroma samples, but also because of the amount of bacterial DNA lost during the extraction process. Nested-PCR is a modification of conventional PCR normally used when a low bacterial load is expected (47). A threshold detection level of ~3000 bacterial cells/mg of tissue was established in this study, and this molecular diagnostic system allowed the investigators to detect the five target periodontopathogens in atheroma samples (P. gingivalis, T. forsythia, P. intermedia, A. actinomycetemcomitans, and F. nucleatum).

As mentioned above, some previous studies (See Table 1 and 2) aimed to identify the simultaneous presence of some periodontopathogens in subgingival and atheroma samples. Three of these studies (31,36,38) found no periodontopathogens in atheroma samples by simple PCR amplification. Other studies (32,35,37,40,43,45-47) demonstrated the simultaneous presence of some bacteria in both locations, but few (43,45,47) evaluated the statistical power of this finding, although no statistical significance was shown. More recent findings (33) demonstrated the presence of A. actinomycetemcomitans, P. gingivalis, T. forsythia in both locations, but only P. gingivalis showed a statistically significant correlation. Marcelino et al. (42) observed a statistically significant association between the presence of T. forsythia and P. gingivalis, and periodontal pockets ≥5 mm. Mahendra et al. (41) revealed a significant correlation between the plaque index, clinical attachment level and probing depth and the presence of T. denticola in atherosclerotic plaques. Other correlated markers of cardiovascular diseases were described by Koren et al.44 who demonstrated that Fusobacterium ssp. and Streptococcus ssp. were positively correlated with high levels of cholesterol.

Given that a high percentage of patients (45%) showed a simultaneous presence of P. gingivalis in periodontal pockets and atheroma plaques, our findings are in agreement with Ishihara et al. (33) and Marcelino et al. (42). Moreover, the results of the present study also detected a high frequency of F. nucleatum and T. forsythia in the periodontal pocket and in the atheroma (p-value <0.1). These findings support the theory that atheroma colonization is not only restricted to P. gingivalis, but also to other periodontopathogens. It must also be taken into account that a quantitative method of detection would be more likely to
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The association between high serum glucose levels and the presence of P. intermedia in atheroma samples has led us to hypothesize that an environment rich in glucose might help the metabolism of these oral bacteria and their persistence in the vascular endothelium. The pathogenic mechanisms of P. intermedia are not fully understood, but some studies have demonstrated a bidirectional link between diabetes mellitus and periodontitis (17,75). Another important finding was the relationship between elevated lymphocyte serum levels and high BI, and the detection of the five target periodontopathogens in atheroma. This link was not demonstrated with other acute phase cells, like neutrophils. These results are in accordance with Zaremba et al. (37), who found higher BI in patients with bacteria in atheroma and no differences of neutrophil levels among patients. Furthermore, the subacute inflammation pattern was the most prevalent found in the atheroma biopsies, suggesting a local active inflammatory process in the atheroma plaques. Our findings strengthen the hypothesis that the persistence of a chronic inflammation produced by a non-controlled periodontitis might have a negative effect on the intravascular endothelium and on the development of atherosclerosis, due to the recurrent input of inflammatory biomarkers (76). Therefore, no concrete association could be drawn on these findings, further research is needed to confirm these results.

Finally, Epstein (77) showed that the bacterial load in subgingival periodontal pockets might act as a reservoir for the microbiological environment, and promote the development of atherosclerosis. The results of our study define the load of F. nucleatum, T. forsythia and P. gingivalis in the possible development of the atheroma plaques. On the other hand, the predominant presence of A. actinomycetemcomitans, an anaerobic facultative pathogen, and the high frequency of P. intermedia in the atheroma, seems to be unrelated to the bacterial load in the subgingival pocket, but once these bacteria have colonized the vascular endothelium, they are able to remain there for a long period of time, maybe helped by high serum glucose levels.

With the data obtained in this study, we conclude that the presence of P. gingivalis, F. nucleatum, and T. forsythia in subgingival plaque suggests that these bacteria might significantly contribute to the development of atherosclerosis. Alterations in some biochemical parameters might promote bacterial colonization and metabolism, and therefore contribute to the development of atherosclerosis.

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