“In vitro” Studies Linking Atherosclerosis and Periodontitis.

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Abstract

Background: Atherosclerosis and periodontitis are chronic inflammatory diseases that share risk factors and have been linked in several studies, but mechanisms of this association remain controversial. This review aimed to ascertain the current status of the relationship between atherosclerosis and periodontitis, based on studies designed to detect bacterial DNA on cardiovascular specimens.

Methods: An electronic and manual literature search in MEDLINE via PubMed, Biosis via Ovid, Lilacs and the Cochrane Library databases between January 1995 and December 2013 was conducted. Only studies analyzing periodontal bacteria detection in cardiovascular specimens and/or in periodontal pockets were included. Descriptive analysis was used to calculate the absolute frequency of each group of articles (Group A and B), in vitro detection methods and number of analysed bacterial species.

Results: Thirty-two out of 378 epidemiological studies were included in this review and classified into two groups: papers analysing periodontopathogens from oral biofilm and cardiovascular specimens (n=17; Group A), and papers analysing periodontopathogens only in cardiovascular specimens (n=15; Group B). The most relevant relative frequency of bacterial detection in cardiovascular samples in Group A was 26% P.g, 14% F.n, 12% A.a, 8% Pi, 6% T.f, and in Oral samples was 78% P.g, 68% F.n, 65% P.i, 65% T.f, 27% A.a. In Group B the relative frequency was 30% P.i, 20.1% P.g, 17% F.n, 16.53% A.a, 13.17% T.f.

Conclusions: Although the present data show a trend to the presence of some periodontopathogens in atheromatous plaque, supporting the possible hypotheses that might explain the association between periodontitis and atherosclerosis, further investigations are needed considering the high number of common risk factors in order to clarify the association between both diseases.

Keywords: Atherosclerosis, cardiovascular diseases, chronic periodontitis, oral biofilm

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Introduction

Cardiovascular diseases (CVD) including atherosclerosis are the leading cause of death in many societies. CVD accounts for an estimated 40% of all deaths worldwide, with atherosclerosis being the primary aetiology (1). Besides the high prevalence of CVDs, periodontitis is also a widespread infectious-inflammatory disease (2,3), which is considered the most prevalent chronic condition in humans.

Several authors have reported that chronic periodontitis might significantly contribute to the progression of certain systemic diseases (4-7), such as respiratory (8,9) diseases, diabetes mellitus (10,11), preterm low-weight births (12,13), hyperlipidemia (14,15), osteoporosis (16) and cardiovascular diseases (17-21). Periodontitis has been linked to atherosclerosis in a relevant number of investigations (4,22-24). The two diseases, which have many pathogenic mechanisms in common (22,23,25-29) and a complex aetiology, share many common risk factors such as age, education, social status, stress, smoking, obesity and hypoglicemia (22,23,30). These hypotheses have been based on evidence that some bacteria in the subgingival biofilms may alter the resulting host response involved in the pathophysiology of the atherosclerotic lesion (23,27).

The possible mechanisms that increase inflammatory responses in atheromatous lesions due to periodontal infections may include a rise in systemic levels of inflammatory mediators stimulated by bacteria and their products at sites distant from the oral cavity (31), as well as elevated thrombotic and haemostatic markers that promote pro-thrombotic states and inflammation (32,33). Other causes that could promote inflammation and interact with the atheroma are the cross-reactive systemic antibodies (34), the promotion of dyslipidemia (35,36), and common genetic susceptibility factors present in both diseases that could lead to increased inflammatory responses (37,38).

Although different methods have been used to detect and quantify putative periodontal pathogens, no detection method has demonstrated ideal characteristics. In addition, procedures may vary significantly according to atheromatous plaque collection and process system. For these reasons, the aim of this study was to evaluate the available information on molecular techniques to identify periodontal bacterial DNA in cardiovascular specimens.

Methods

Search strategy

A systematic electronic and manual literature search covering the period between January 1995 and December 2013 was conducted to find studies dealing with possible associations between periodontal disease and atherosclerosis.
The following databases were used: MEDLINE via PubMed, Biosis via Ovid, Lilacs and the Cochrane Library. Only studies analysing periodontal bacteria detection in cardiovascular specimens and/or in periodontal pockets were included in this review. Papers that did not analyse periodontal pathogens or that were not written in English were excluded from the present literature review. Abstracts were scanned to select only original research into periodontitis and detection of periodontopathogens in cardiovascular specimens (Figure 1).

Two independent reviewers screened full-text articles. Original clinical studies, with an objective of detecting periodontal pathogens in cardiovascular specimens and/or periodontal samples using in vitro bacterial detection methods, were considered for inclusion (Inclusion-Exclusion criteria). Any disagreement was resolved by consensus.

The criterion to consider a specific bacterium as a potential periodontopathogen was based on the complexes proposed by Socransky (39).

Selected full text articles were divided into two main groups. Group A (GA) were papers analysing periodontopathogens from oral biofilm and cardiovascular specimens, and Group B (GB) were papers analysing periodontopathogens only in cardiovascular specimens. Each main group (GA and GB) was divided into two subgroups based on the provision (Subgroup 1) or not (Subgroup 2) of complementary data such as periodontal status and medical condition of each patient.

Statistical analysis
Descriptive analysis was used to calculate the absolute frequency of each group of articles (Group A and B), in vitro detection methods and number of analysed bacterial species. Detection frequency of each target pathogen in dental and atheromatous samples was also obtained. Relative frequency of bacterial detection was calculated to obtain information about frequency of bacterial detection related to the number of samples in each investigation.

Results
A total of 378 references were identified by keyword search from the above-mentioned databases. The identified articles were retrieved and the abstracts screened to select only original clinical studies whose aim was to detect periodontopathogens in cardiovascular specimens. The search resulted in a total of 32 studies (40-57,59-72) containing inclusion criteria. Each full text of the selected studies was reviewed and classified into one of the two proposed groups (Table 1). A comprehensive methodological quality assessment was carried out of the included studies:

Sample size: The variability of the sample size and the relatively small number of subjects for statistical analysis due to the circumstances surrounding specimen collection.

Control group: Due to ethical reasons, most of the investigations had no control group. It should be noted that the three studies (52,55,59) containing samples without atherosclerotic findings as a control group were specimens that had undergone an atherosclerotic process. One study (54) compared two different locations (heart valve and atheromatous plaque specimens), while the majority of studies (40,51,60) included different periodontal conditions or edentulous patients in order to compare the detection rate in atheromatous specimens.

Correlation of samples: It was observed that nearly half of the investigations studied the presence of periodontal pathogens only in atheromatous plaque without determining bacterial composition in the oral sample in
order to compare the two locations and demonstrate a positive correlation. Furthermore, most of the studies that detected periodontopathogens in cardiovascular specimens and in dental plaque did not describe the relationship between the two samples by pairs (patient by patient) (41,42,46,48,49,50-53,56). By contrast, some articles (43-45,51,55,59-61,63,65,70) showed the concomitance of periodontal bacteria in atheroma plaques but few of them (43-45) described the association between the presence of periodontopathogens in atheroma plaques and dental plaque.

Medical and periodontal conditions: Data-collection of the patients’ medical and periodontal conditions was limited. Occasionally data regarding the presence of certain risk factors such as age, education, social status, stress, smoking, obesity and periodontal examination to evaluate the medical condition or the degree of periodontal disease was recorded (40,41-48,50-53,57,59). 38% of the investigations (49,54,56,64-72) did not record any medical and periodontal parameters. In other cases (40,41,45,47,49,52,59,60,62), this information was not related to bacterial detection.

Processing samples: Because of the low bacterial load in atheroma specimens, DNA detection was an additional limitation and strict laboratory protocols are needed (73,74). In the analysed studies, the processes of specimen collection and DNA extraction were not usually reported (46,49,56,64). Furthermore, the amount of atheromatous plaque specimens also varied among the studies or the source was not specified (41,43,44,46,52,54,56,59,61-67,70).

Detection methods: Although different methods of microbial diagnosis have been used to detect and quantify putative periodontal pathogens, there is no single method that has demonstrated ideal characteristics (75). The majority of the investigations have used qualitative PCR technology as the diagnosis method. Four out of 30 articles used real-time PCR, which has a high degree of sensitivity and specificity, and provides accurate information on the number of cells of each species identified, which is crucial to molecular diagnosis (75).

Origin of the specimen: The reviewed papers showed differences in the bacterial species detected and their bacterial load depending on the location of the cardiovascular specimen that was analysed (76). This approach could affect the results by giving rise to possible false negative bacterial detections due to the distance between the periodontal pocket and the atherosclerotic lesion.

According to the stratification proposed in this study, 53% of the articles (n=17) aimed to detect periodontopathogens from both oral and cardiovascular samples (Group A). On the other hand, 47% of the articles (n=15) only aimed to analyse cardiovascular specimens (Group B).

The different techniques used in the papers to detect bacterial DNA are included herein. 43% of the authors chose the qualitative PCR as a detection tool, making it the most common method. Variants of the PCR technique, such as nested-PCR or real-time PCR, were applied in 6% and 16% of the studies, respectively. Complementary methods, such as DNA-hybridization (16%), immunofluorescence (3%), culture (9%) or histological assay (3%), were used to a lesser extent.

Studies from Group A

This group contained 17 articles (40-56), which analysed periodontopathogens in atheromatous specimens and subgingival samples from the same patients. Fourteen studies (40-53) (83%) analysed medical and/or periodontal conditions, whereas
three of them (54-56) (18%) did not evaluate any medical and/or periodontal data of patients included in the samples. PD was the parameter of choice when periodontal examinations were performed (76,47%). Eight studies (40,44-46,48,50) registered CAL and seven studies (40,44-46,48,50,51) included BP, PI and number of missing teeth. Only three articles (40,44,51) used radiographic methods in order to determine periodontal bone lost. Regarding patients’ medical conditions, 11 out of 14 articles (40,41,43-48,51-53) evaluated some medical data, of which smoking was the most recorded parameter (seven out of 11 articles (40,41,43-47,52-53)), followed by diabetes and hypertension (six out of 11 (40,41,47,48,52,53)). Other data, such as presence of CVD or dyslipidaemia, were registered in five (40,43,47,51-53) and four (41,43,52) articles respectively. Only four articles (41,43,46,53) evaluated biochemical parameters such as blood cell count, blood chemistry, coagulation test, and triglycerides levels.

The most frequently analysed pathogen in the two samples (subgingival plaque and cardiovascular specimens) was Porphyromonas gingivalis (P.g) (100%), followed by Aggregatibacter actinomycetemcomitans (A.a) (88%), Tannerella forsythia (T.f), Treponema denticola (T.d) and Prevotella intermedia (P.i) (82%), Campylobacter rectus (C.r) (47%), Fusobacterium nucleatum (F.n) (41%), Prevotella nigrescens (P.n) (18%) and Eikenella corrodens (E.c) (12%). Regarding bacterial detection in cardiovascular specimens, three investigations (40,45,47) found no bacteria in carotid atheromatous plaques using specific PCR, nested-PCR, and reverse hybridization techniques respectively. On the other hand, some studies (41-44,46,48-53) detected periodontopathogens in their cardiovascular specimens. Figure 2 shows the frequency of bacterial detection in oral and cardiovascular samples.

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Figure 2. Relative frequency of bacterial detection in articles analyzing periodontopathogens from oral biofilm and cardiovascular specimens (Group A).

The relative frequency of bacterial detection in cardiovascular samples was 12% A.a, 26% P.g, 8% Pi, 6% T.f, 14% F.n, 28% T.d, 13% C.r, 20% E.c, 10% P.n and in oral samples was 27% A.a, 78% P.g, 65% P.i, 65% T.f, 68% F.n, 60% T.d, 55% C.r, 64% E.c, 24% P.n.

Studies from Group B

A total of 15 articles (57,59-72) were included in this group, which analysed periodontopathogens only from atheromatous specimens. Six articles (57,59-63) (40%) evaluated medical and/or periodontal conditions, while 60% of the studies (64-72) did not evaluate any medical and/or periodontal data of patients included in the samples. All studies recorded periodontal parameters and some of them took into account the number of lost teeth (57,60-62) (33.33%) and PD60 (20%). Other collected data included CAL: BP and gingival recession (59,60,62) (13.33%) and dental mobility (61) (6.66%). Only one article (61) used radiographic methods in order to determine bone lost. Regarding patients’ medical conditions, only five articles (57,59,60,62,63) (33.33%) recorded smoking habits, diabetes, hypertension or presence of...
CVD. Moreover, few articles included mean body mass (59), biochemical parameters (59) and education (62).

The most frequently analysed pathogens in cardiovascular samples were P.g (100%), followed by A.a (86%), T.f (67%), P.i (40%), P.n (27%), F.n and C.r (20%), E.c and T.d (13%). As for bacterial detection in cardiovascular specimens, one investigation (62) found no bacteria in atheromatous plaque at different locations using PCR techniques. All other studies (57,59-61,63-72) detected some periodontopathogens in their cardiovascular specimens. Figure 3 shows the frequency of periodontopathogens in cardiovascular specimens.

**Figure 3. Positive periodontopathogens detection in articles analyzing periodontopathogens only in cardiovascular specimens (Group B).**

The relative frequency of bacterial detection was 16.53% A.a, 20.1% P.g, 30% P.i, 13.17% T.f, 11.4% T.d, 26.9% E.c, 11.38% P.n and 2.72% C.r.

**Discussion**

Atherosclerosis and periodontitis are chronic inflammatory diseases that share some risk factors and have been linked in several studies (19,24,77). Some hypotheses were proposed to explain why periodontitis may increase the risk of CVDs (22,24), from an indirect association determined by common risk factor or phenotype to a direct association between the periodontal infection and the pathophysiology of the atherosclerotic lesion (29,78). This last hypothesis was supported by evidence that bacterial pathogens derived from the subgingival biofilm might be directly or indirectly involved in the process of atherogenesis (19).

For this reason, detection of periodontal bacteria DNA in cardiovascular specimens has been one way to establish a possible association between periodontitis and atherosclerosis. Differences in study designs and methodologies may be responsible for conflicting results published in the scientific literature.

The evaluation of these studies shows a high variability of the sample size and the relatively small number of subjects due to the circumstances surrounding specimen collection and these were not “true control groups” because the patients may have had periodontal disease as well. These limitations make it difficult for a statistical analysis and interpret results.

Another fact is the lack of simultaneous microbiological analysis in the two locations (oral and cardiovascular specimens) that difficult the understanding of the role of periodontitis in atheroma plaque formation. Therefore, the ideal design for these types of studies would be a simultaneous detection in the two locations correlating the presence of periodontopathogens in each subject.

The medical and periodontal data allowed us to make a multifactorial analysis of common risk factors. The absence of these data in some investigations hinders the evaluation of periodontitis as a potential risk factor because this condition may be a "confounding factor". It would be necessary in these types of studies to record all
common risk factors and carry out a multivariate analysis to determine the importance of periodontitis as a possible factor related to atherosclerosis lesions. From a public health point of view, and taking into account periodontitis as a factor involved in atherosclerosis, periodontal treatment should be included in order to improve atherosclerosis development.

Taking into account that DNA extraction from atheromatous plaque is a complex process, it is essential to describe complete protocols for managing the sample (from sample collection to bacteria detection) in order to detect possible deficiencies in the process that may determine the results of bacterial detection.

It is important to emphasize that the presence of bacterial DNA in atheromatous plaque remains a controversial issue (42). The detection of pathogens may vary according to both the chosen portion of the atheromatous plaque (76) and the diagnostic method used to obtain false-negative presence in the sample. In a multicentre PCR comparison trial to detect Chlamydia pneumoniae (C.p) in endarterectomy specimens, the positivity rate varied between 0 and 60% using different test methods in the same atheroma (73). Ideally, to detect the possible presence of the bacteria in the atheromatous lesion, as well as bacteria viability, the cardiovascular specimen should be taken as close as possible to the periodontal pocket, preferably in the region of the carotid artery.

The periodontopathogens most frequently analysed were P.g, A.a, T.f, P.i and T.d in concordance with those described by Socransky (39) strongly related to periodontal disease. The most detected periodontopathogens in Group A, in order of frequency, in oral samples were P.g, F.n, P.i, T.d and in atheromatous lesions were T.d, P.g, E.c, F.n, A.a, whereas three (40,45,47) investigations observed no bacteria in atheromatous plaques. These findings reveal that only P.g showed a correlation between two locations. There is an interesting discordance among the pathogens from the oral samples and the pathogens in cardiovascular specimens. Most of the studies did not offer any data about the bacteria concomitance between both samples, thus it was difficult to obtain a comparison and to correlate the results. In group B, the most detected periodontopathogens were P.i, E.c, P.g, F.n, A.a, and 4 out of 32 investigations (40,45,47,62) found no periodontal pathogen in atheromatous plaque. These heterogeneous results might be put down to a lack of a standardized protocol in the methodology of cardiovascular specimen analyses as well as factors, such as the cell invasion ability of bacteria (27), that are significant for detecting bacteria in atherosclerotic plaque. The behaviour of each bacterium in terms of invasion, propagation and colonization of distant tissues needs to be studied in order to understand the presence of periodontopathogens in cardiovascular specimens.

These findings highlight the importance of choosing a good diagnostic method in vitro to ensure proper detection of pathogens in samples. Future research should use quantitative diagnostic methods and discriminate between live and dead cells. This information would have a value as an adjunct in order to demonstrate a positive correlation between the patients' periodontal status and the prevalence of periodontal pathogens detected in their cardiovascular specimens, thus contributing to a clinical diagnosis and treatment planning in the two diseases.

Finally, discrepancies among investigations may be explained by ethnic differences or geographic factors in the populations under study. Differences in subgingival microbial species and host immune response have been reported in various racial groups (79). An alternative hypothesis could be that the prevalence of
periodontal bacteria DNA in atheromatous lesions differed for epidemiological reasons.

**Conclusion**

In conclusion, many studies have identified periodontopathogens in cardiovascular samples trying to explain a possible way of association between periodontal diseases and atherosclerosis. However, the mere presence of periodontal bacterial DNA in atheromatous plaque does not imply a direct relationship between both diseases.

These studies, on account of their low number of subjects and lack of control group, prevent a correct statistical analysis, as it is difficult to correlate values and a correct validation of results. The heterogeneity of study design and methodologies in terms of sample number, medical and periodontal data collection, bacterial detection methods and the results of the investigation show the need for a consensus to optimise bacterial detection methods and to conduct a reproducible protocol to standardise these types of studies.

Although the present data show a trend to the presence of some periodontopathogens in atheromatous plaque, supporting the possible hypotheses that might explain the association between periodontitis and atherosclerosis, further investigations are needed considering the high number of common risk factors in order to clarify the association between both diseases.

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