The Evaluation of the C Reactive Protein Levels in the Context of the Periodontal Pathogens Presence in Cardiovascular Risk Patients

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The purpose of this study was to investigate the serum C-reactive protein (CRP) values in the presence of A. actinomycetemcomitans, P. gingivalis, T. denticola or T. forsythia bacteria, as an indicator of the cardiovascular risk. The study consisted of 64 male and female subjects, aged 55 to 75 years. Subjects were periodontal examined, serum CRP was analyzed, and Porphyromonas gingivalis, A. actinomycetemcomitans, T. forsythia and T. denticola from the subgingival bacterial plaque were detected by real-time quantitative polymerase chain reaction (qPCR). Pathogen prevalence rates were: 45.0% P. gingivalis; 20.5% A. actinomycetemcomitans; 86.1% T. forsythia; 86.3% T. denticola. The mean CRP was 1.5 (IQR 1.0-2.6) mg / L. There was a significant difference in CRP values between subjects who had P. gingivalis compared to those without (p = 0.003). There were no significant differences for any of the other pathogens. The presence of P. gingivalis was associated with a 1.20-fold increase in CRP. Of the four periodontal pathogens investigated, only the presence of P. gingivalis in subgingival plate samples was significantly associated with a high level of C-reactive protein.

Keywords: C reactive protein, parodontopathogenic bacteria, cardiovascular risk

Chronic periodontitis is caused by pathogenic microorganisms present in mature biofilms - bacterial plaque. Bacteria identified as most likely to play an etiological role in the development of periodontal disease include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* [1]. The periodontal pocket is a niche that hosts hundreds of bacterial species, including those identified as periodontal pathogens, resulting in a chronic inflammatory site with ulcerative epithelium, which is an entry point for bacteria and / or their by-products in the systemic circulation [2]. The net result is the activation of the host's inflammatory response through multiple mechanisms.

C Reactive Protein (CRP), a protein mainly produced by the liver in response to an increase in interleukin-6 and tumor necrosis factor alpha, is part of the non-specific response to inflammation, infection and tissue damage [3]. It has been shown in several prospective epidemiological studies that CRP is an independent predictor of cardiovascular disease [4] and is considered a biomarker of systemic inflammation. There is strong evidence that individuals with chronic periodontitis have elevated CRP levels compared to the control groups [5]. There is also moderate evidence that periodontal therapy decreases serum CRP [6].

Atherosclerosis is considered to be a manifestation of a disordered immunity state in which there is a dynamic interaction between endothelial dysfunction (characterized by loss of normal endothelial vasodilation), inflammation and repeated response cycles for wound healing [7]. Coronary artery disease is a major cause of morbidity and mortality worldwide. Common risk factors such as hypercholesterolemia, smoking and high blood pressure have failed to fully explain the incidence of coronary artery disease, epidemiological studies indicating links between periodontal disease and coronary artery disease [8].

Possible role of infection in atherogenesis has been postulated, involving several pathogens, some playing a more prominent role (eg *P. gingivalis, Chlamydophila pneumoniae*), where the risk of coronary artery disease refers to the total pathogenic total burden [8]. Specifically, Pussinen et al. [9] concluded that a high level of anti-P. gingivalis antibodies, frequent in patients with periodontal disease, has been associated with coronary heart disease and resulted in an increased risk (approximately 1.5 times higher) for coronary risk after adjustment for various common risk factors. In addition, Tang et al. [10] found that the risk increases with the severity of periodontitis, so patients with severe periodontal disease have an increased risk of 2.0 times to develop coronary heart disease. In addition, infection may also trigger thrombotic plaque rupture and acute thrombotic occlusion, major factors responsible for acute myocardial infarction and sudden death in patients with coronary artery disease [8].

It has been hypothesized that there is a complex relationship between periodontal disease and increased risk of acute myocardial infarction [11]. Mechanisms are now under development in support of this hypothesis, linking transient bacteria, frequently in periodontal disease, with endothelial dysfunction, an event in the development of atherosclerosis [12]. Periodontal infection induces local inflammation and several studies have demonstrated that patients with periodontal disease have high levels of systemic inflammatory mediators, such as C-reactive reactive protein (CRP) [13]. This inflammation often leads to gingival ulcerations and local vascular changes, which have the potential to increase the incidence and severity of transient bacteremia.

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The purpose of this study was to investigate whether the presence of *A. actinomycetemcomitans, P. gingivalis, T. denticola* or *T. forsythia* in a group of patients aged 55-75 years was associated with systemic inflammation quantified by the values Serum levels of CRP.

Experimental part

The study consisted of 64 male and female subjects aged 55 to 75 years. Subjects were periodontally examined, with the determination of the probing depth, the periodontal attachment level, the number of present teeth (except the wisdom molars), with the diagnosis of periodontal disease.

Subgingival bacterial plaque sampling was performed from sites with the highest depth found in the patient. The sites of interest were isolated with cotton rolls and gently dried with the air spray. The bacterial plaque was harvested using a single Gracey curette (Hu-Friedy, Chicago, IL, USA) from the base of the pocket to the coronary side. The samples were then placed in phosphate solution and immediately transferred for storage at -80^{0}C until analysis. *P. gingivalis, A. actinomycetemcomitans, T. forsythia* and *T. denticola* were detected by real-time quantitative polymerase chain reaction (qPCR). Subjects were classified as having each pathogen present or not; this was an aggregate of all plaque samples, which means that if at least one sample for a subject was positive, the subject was reported to be positive for the pathogen.

For determination of C-reactive protein, venous blood samples were harvested and centrifuged. Small quantities were then frozen and stored at -80^{0}C until analysis. CRP was measured using Quantex Biokit Reagents.

Body Mass Index (BMI) was calculated as weight / height^2 (kg / m^2). Smokers have been classified as current smokers or not. Diabetes and hypertension were determined by specific measurements (blood glucose and blood pressure, correlated with patient history). A history of cardiovascular disease was recorded for subjects who had a previous myocardial infarction or an intervention such as angioplasty or bypass with stenting of the coronary arteries. Cerebral-vascular disease was recorded for subjects who had previously had a stroke. Material conditions were classified according to the type of home and lifestyle (rented or owned / mortgaged), the number of cars / vans / motorcycles in the household and the number of bathrooms and / or showers and toilets in the house.

Data for CRP values was not normally distributed, therefore the log converted values were used for analysis. Independent t tests were used to compare the transformed log means of CRP values based on the presence or absence of each separate pathogen. The association of each pathogen with both moderate and severe periodontal disease was analyzed by the chi-square test (Yates correction). Multiple regression analysis was performed in a sequenced design model in which categories of potentially complex variables were added to produce a fully-tailored final model for CRP-independent predictors. The significance level for all assays was set at $p < 0.05$. Statistical analyzes were performed with SPSS version 21 (IBM Corp, Armonk, NY, United States).

Results and discussions

Based on the statistical analysis, there were no significant differences in age, BMI, smoker status or CRP. The mean age of the 64 subjects was 72.5 years, with a range of 55-75 years. Mean BMI was 27.3 kg / m^2, with 21% of subjects classified as obese (BMI ≥ 30 kg / m^2). 21 subjects (30.40%) had mild periodontitis, 32 subjects (50.00%) had moderate periodontitis, and the remaining 11 subjects (19.60%) had severe periodontitis. These and other characteristics of the subjects studied are presented in table 1. The prevalence rates of the pathogens were: 45.0% *P. gingivalis*; 20.5% *A. actinomycetemcomitans*; 86.1% *T. forsythia*; 86.3% *T. denticola*. The median CRP was 1.5 (IQR 1.0-2.6) mg / L.

We analyzed the risk levels according to CRP. Following this analysis, 11 subjects (19.6%) had a low risk (<1.0 mg / L); 15 (23.4%) have a medium risk (1.0-3.0 mg / L); and 38 subjects (57.0%) were in a high risk category (> 3.0 mg / L). There was a significant difference in CRP values between subjects who had *P. gingivalis* compared to those who did not ($p = 0.003$). There were no significant differences for any of the other pathogens (table 2). There was a significant association ($p < 0.001$) for each of the four pathogens investigated with moderate periodontitis. The differences were also significant for severe periodontitis: *P. gingivalis* ($p = 0.01$), *T. forsythia* ($p < 0.01$) and *T. denticola* ($p < 0.01$), all associated with periodontitis, but not for *A. actinomycetemcomitans* ($p = 0.12$), as shown in table 3.

Multiple regression analysis showed that the body mass index ($p < 0.001$), current smoking ($p < 0.01$), hypertension ($p = 0.01$) and the presence of *P. gingivalis* ($p < 0.01$) are independent CRP predictors.

The presence of *P. gingivalis* was associated with a 1.20-fold increase in CRP (95%, confidence interval 1.04-1.37) in the fully-adjusted model. There were no significant associations between the presence of other periodontal pathogens investigated and CRP.

The main finding of this study was that the presence of *P. gingivalis* in the subgingival plate was significantly associated with the C-reactive protein level in a homogeneous group of 55-75 year-olds. This relationship remained significant after adjusting for various bias factors. There were no associations between the presence of several other periodontal pathogens and the level of CRP.

The bacterial species identified in the *red complex* [14], together with *A. actinomycetemcomitans*, are frequently isolated together and have been strongly associated with periodontal disease [1]. Our hypothesis was that these

Table 1

CRP VALUES BASED ON THE PRESENCE OR ABSENCE OF THE INVESTIGATED PATHOGENS

	Present pathogen		Absent pathogen		p-Value
	n	CRP (mg/l) Mean	n	CRP (mg/l) Mean	
P.gingivalis	30	2.03 (1.2-3.08)	34	1.53 (0.88-2.32)	0.003*
A.actinomycetemcomitans	12	1.87 (1.04-2.84)	52	1.70 (1.02-2.60)	0.82
T.forsythia	54	1.88 (1.15-2.64)	10	1.87 (0.86-3.60)	0.91
T.denticola	56	1.89 (1.14-2.76)	8	1.80 (1.05-2.61)	0.59
*Statistical significance (p<0.05)					

Table 2
PREVALENCE OF MODERATE / SEVERE PERIODONTITIS DEPENDING ON THE DETECTED PATHOGEN

	Periodontitis prevalence		Odds ration	(95% IC)		p-Value
	Present pathogen	Absent pathogen				
Moderate periodontitis						
P.gingivalis	11	7	2.52	1.72	3.70	<0,001
A.actinomycetemcomitans	5	13	2.10	1.33	3.30	<0,001
T.forsythia	17	2	1.63	1.63	6.24	<0,001
T.denticola	18	1	2.73	2.73	14.03	<0,001
Severe periodontitis						
P.gingivalis	5	4	1.75	1.17	2.82	0.01
A.actinomycetemcomitans	2	6	1.48	0.84	2.56	0.12
T.forsythia	8	1	4.17	1.37	11.65	<0.01
T.denticola	9	1	5.63	1.62	17.22	<0.01

Table 3
GROUP CHARACTERISTICS DEPENDING ON THE PRESENCE OF *P.GINGIVALIS*

	P.gingivalis present	*P.gingivalis* absent	p-Value
Age (yrs) (mean)	73.8	73.2	0.02*
Present teeth (mean)	18.1	19.00	0.08
BMI (kg/m^2) (mean)	26.3	26.1	0.52
Cholesterol (mmol/l) (mean)	5.7	5.7	0.98
Diabetes mellitus (n)	3	2	0.58
Arterial hypertension (n)	7	2	0.89
Smokers (n)	5	2	0.03*
*Statistical significance (p<0.05)			

periodontal pathogens could also be associated with systemic inflammation, measured by CRP. The study confirmed this hypothesis. It was noted that although all four parodontopathic agents were associated with moderate and severe periodontitis (except *A. actinomycetemcomitans*), only *P. gingivalis* was associated with CRP levels.

A pattern of pathogenesis of periodontitis has been described - *The key pathogen hypothesis* - suggests that the abundance of microbial pathogens such as *P.gingivalis* can orchestrate periodontal inflammatory disease by remodelling the symbiotic microbiota, normally benign to a dysbiotic form [15].

Data from the Human Microbiome Project [16] suggests that there is significant diversity in the microfilm of both healthy and diseased periodontium. Much of the research so far supports *P.gingivalis* as a key pathogen. The murine initial model showed that *P.gingivalis* could trigger changes in the amount and composition of oral microbiota, leading to periodontal bone inflammatory changes [17]. The main identified pathogenic way was the subvertment of the complement, which led to the creation of a dysbiotic microbiota with the clinical signs associated with the disease. This has also been demonstrated in rabbits [18], where *P. gingivalis* caused the transition to an anaerobic microbiota and a global increase in bacterial load. Dysbiotic bacterial load may be of greater importance when analyzing a systemic inflammatory response than the presence of specific pathogens. The broad concept was that the number of different pathogens to which an individual was exposed was an important factor in promoting a synergistic inflammatory response that exacerbated atherosclerosis more than single pathogen infection [8].

In this study, the hypothesis was whether the current exposure to a pathogen in a cross-sectional model is associated with a high level of systemic inflammation. Exposure was defined by the detectable presence of one of the four pathogens. A possible additional explanation for the association between *P. gingivalis* and elevated CRP values may be found in genetic variants in subjects, which may predispose the development of a dysbiotic microbiota and increase the production of pro-inflammatory cytokines. Nibali et al. [19] showed that genetic polymorphisms of interleukin-6 were associated with increased detection rates of *A. actinomycetemcomitans, P. gingivalis* and *Tannerella forsythia*, after adjusting for age, ethnicity, smoking and the severity of periodontitis.

Periodontal disease may, in fact, be just one of the few co-morbidities that develop on the basis of the interaction between microbial dysbiosis and other established risk factors, such as smoking. A good knowledge of the genetic basis of the interaction between the host and the microbe will be necessary to fully understand such mechanisms.

CRP was the main marker investigated in this study, being recognized as an important biological marker of inflammation. Elevated levels of CRP have consistently been shown to increase the risk of cardiovascular disease [20] and of type 2 diabetes [21]. CRP is a non-specific marker of acute phase response, and there are many stimuli, including smoking and obesity, that can induce modest increases in CRP [22]. A very high CRP level (> 10 mg / L) has been reported to reflect acute inflammation, with multiple measurements being required to realistically quantify the degree of inflammation [23].

An important criterion for exclusion was the use of statins. Statins were primarily prescribed for the reduction of low-density lipoprotein cholesterol (LDL) and thus to reduce the risk of cardiovascular disease. However, there is evidence that statins reduce CRP levels in a manner largely independent of LDL cholesterol lowering [24].

The incidence of atherosclerosis cannot be fully explained by classical risk factors [25]. Consequently, the importance of infections as a potential cause of atherosclerosis has gained ground, supported by an abundance of epidemiological evidence supporting this notion [26]. Infectious agents, including periodontal bacteria, have been implicated in the etiology of various vascular conditions through multiple mechanisms, including direct microbial invasion of endothelial cells.

In a clinical context, it is extremely difficult to determine the determinant factor of atherosclerosis for several reasons. First, the initiation factor is likely to be missed since the early phase of the endothelial lesion is usually asymptomatic [27]. Second, atherosclerotic lesion is a common inflammatory response to several factors [28, 29], and some or all of these factors may be associated with the lesion. Thirdly, interventional studies evaluating the impact of periodontal treatment, with or without antimicrobial therapy, on systemic inflammation or endothelial dysfunction, showed mixed results, including lack of changes, transient worsening of signs immediately after treatment, or improvement in signs that did not persist in time [30, 31]. Despite these limitations, invasion of cardiovascular tissues by periodontal bacteria may have the potential to promote atherosclerosis.

Conclusions

Of the four periodontal pathogens investigated, only the presence of *P. gingivalis* in subgingival bacterial plaque samples was significantly associated with a high level of C-reactive protein. This relationship remained significant after adjusting for different bias factors. Knowledge and understanding of the relationship between oral microbiota and both periodontal and systemic health will need to be further developed to fully elucidate the mechanisms of potential associations.

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