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### The clinical, microbiological and systemic characteristics of periodontitis and their changes after periodontal therapy

Bizzarro, S.

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# CHAPTER 1

## **General introduction**



Periodontitis is a destructive inflammatory disease of the supporting tissues of the teeth, which leads to connective tissue attachment and alveolar bone loss, and eventually, in severe cases, loss of teeth (Pihlstrom et al. 2005). Epidemiological studies show that about half of the population is affected by periodontitis (Albandar 2011, Eke et al. 2015, Eke et al. 2012, Hugoson & Norderyd 2008, Norderyd et al. 2015) and that severe periodontitis has a prevalence of 8-15% (Albandar & Rams 2002, Demmer & Papapanou 2010, Norderyd et al. 2015), with high variability among studies depending on the characteristics of the populations investigated and depending on the definition used.

Periodontitis is a complex disease, meaning that the process of onset and progression of periodontitis involves multiple etiological factors that act simultaneously and lead to a shift from a healthy periodontal status to a diseased status. These factors can be clustered into five major groups: microbiological factors (which involve the colonization of the subgingival environment forming a bacterial biofilm), factors related to the genetics of the host and epigenetic modifications of the genome during life, lifestyle factors (as smoking, stress, diet), systemic conditions (as diabetes mellitus, obesity, metabolic disorders) and other still unknown factors.

Treatment of periodontitis is difficult, time consuming, costly and, moreover, the result is not always predictable. This aspect will be elaborated below.

### **Microbiological etiology of periodontitis**

In the last 50 years, different hypothesis have been formulated in order to explain the role of microorganisms in the onset of the disease (Lopez et al. 2015). The “non-specific plaque hypothesis” claimed that periodontitis is the result of the infection and overgrowth of microorganisms in the subgingival environment which would lead to a change in the proportion of different species (Theilade 1986). As a consequence, the imbalance of bacterial species, which is nowadays known as microbial dysbiosis, would trigger the host response to elicit a local inflammatory response that can eventually result in periodontal destruction. The “specific plaque hypothesis”, on the other hand, claimed that the onset of the disease was mainly related to the colonization of specific pathogenic species, which normally would not be present in healthy conditions (Loesche 1976). Based on this hypothesis, there is the suggestion that bacterial pathogens, by presenting specific antigens or producing enzymes and toxic agents, would be able to trigger the immunological response by the host that would cause eventually periodontal destruction. This hypothesis stimulated researchers to undertake

epidemiological investigations in order to identify bacterial species which would meet these characteristics. As a result, a number of specific microorganisms have been associated to periodontitis. These include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Tannerella forsythia* and *spirochetes* (Socransky & Haffajee 2005).

The two hypotheses discussed above, on the microbial etiology of periodontitis, still could not explain those cases where disease was diagnosed in the absence of “periodontal pathogens”, or clarify why certain subjects could harbor high amount of plaque or show presence of these pathogenic microorganisms without showing any sign of disease. In the early 90’s, Marsh formulated the “ecological plaque hypothesis”, on the basis of which the change in the environmental or host factors (such as changes in microbial nutrient profile in the gingival crevicular fluid, decrease in saliva flow, changes in salivary and pocket pH due to change in diet habits) would determine the shift in the equilibrium between the different bacterial communities (Marsh 1994). Therefore, according to this hypothesis, the interplay between the host factors and the microbial factors and not the mere colonization of pathogenic species, would determine the shift from periodontal health to periodontitis.

More recently the “polymicrobial synergy and dysbiosis model” (PSD) of periodontal disease etiology has been proposed (Hajishengallis & Lamont 2012). According to this latest model, development of disease occurs because of a change in the balance between different bacterial communities, which in turn causes a shift from a symbiotic status to a dysbiotic status. At the base of the PSD model is the polymicrobial synergy among bacteria. To cause dysbiosis, the role of certain “key pathogens” (as *P. gingivalis*) who are capable to either impair the host immune response, or to increase the pathogenicity of other microorganisms, is essential.

### **Treatment of periodontitis based on microbial etiology**

Already in the 18<sup>th</sup> century, Pierre Fauchard understood that the removal of dental deposits would improve the conditions of teeth and gums. In modern periodontics, the first phase of periodontal therapy is composed of the non-surgical removal of supra- and subgingival plaque and calculus deposits, together with a good oral hygiene from the patient. Thus, “basic periodontal treatment”, “initial treatment”, “hygiene phase” or “non-surgical therapy” are all terms that include a therapy that aims to the non-specific disruption of the supra- and subgingival biofilm by means of scaling and root planing (SRP) and oral hygiene instructions.

In the '80s, Badersten and coworkers (Badersten et al. 1987) showed that this therapy, when followed by an appropriate maintenance program, is able to substantially decrease the periodontal inflammation, as evidenced by a strong reduction in the frequency of bleeding on probing, reductions in pocket depth, some clinical attachment gain and to promote, for most patients, a stable periodontal condition. However, despite the evidence of an average efficacy of this non-surgical treatment, there is still a number of patients who show deterioration of their periodontal conditions after treatment (Haffajee et al. 1997). As a possible explanation for these results, microbiological studies proposed the presence of specific microorganisms, which were difficult to be removed by the mechanical debridement. A classic example is the evidence that *A. actinomycetemcomitans*, is capable to invade the periodontal connective tissues (Christersson et al. 1987). The use of non-surgical adjunctive therapies to the basic treatment of periodontitis has been proposed in order to increase the clinical improvements of the non-surgical periodontal therapy and to better remove the periodontal pathogens (Ciancio 1986).

The use of systemic antibiotics as supportive therapy of the basic periodontal treatment has been extensively investigated. The administration of different antibiotic monotherapies as doxycycline, azithromycin and metronidazole, or the combination of metronidazole and amoxicillin have demonstrated an additional improvement of the clinical and microbiological parameters of periodontitis in comparison to the basic periodontal treatment alone (Keestra et al. 2015, Preshaw et al. 2008, Preus et al. 2013, Soares et al. 2014, Winkel et al. 2001).

Next to systemic antibiotics, different local delivered antimicrobials have been also used as adjunctive therapies to the basic periodontal treatment. These include both local antibiotics, such as doxycycline, metronidazole, minocycline, and local disinfectants such as chlorhexidine, povidone-iodine, essential oils and peroxide (Cortelli et al. 2006, Cosyn et al. 2013, Kruck et al. 2012, Leonhardt et al. 2006, Putt & Proskin 2013, Timmerman et al. 1996). However to date, the evidence is inconclusive regarding the real additive effects of these latter therapies, in comparison to the basic periodontal treatment alone (Krayner et al. 2010, Matesanz-Perez et al. 2013).

The indication for the use of systemic antibiotics is still a matter of debate. On one hand, it is suggested that the prescription of these medications should be based on the composition of the subgingival microbiota and more specifically on the presence or absence of specific microorganisms (van Winkelhoff et al. 1996). On the other hand, there is the question whether the presence of a very limited number of bacterial species would be sufficient to determine the

indication for the prescription of antibiotics (Cionca et al. 2009, Mombelli et al. 2013). According to these latter studies, the indication should be evaluated on the single case, based basically on the clinical phenotype of the periodontal conditions and on the therapist's experience. It is clear that the latter approach is debatable since it is based on subjective criteria, which can be difficult to be applied by less experienced clinicians. Nevertheless, the individual-case approach is preferred due to the lack of clear scientifically proven guidelines and also in light of the current emphasis on the danger of antibiotic resistance (Keestra et al. 2015).

### **Microbiological detection techniques**

At the base of the difficulties to solve the debate when antibiotics are indicated, there is the significant drawback from earlier studies that the major part of the microbiological data available are based on the analysis of cultivable species, which however represent only a small part of the total subgingival microbiota, today called "subgingival microbiome". Already in the '90s, Haffajee and Socransky (Haffajee & Socransky 1994) suggested that the immune system in the periodontal pocket is challenged by a mixed infection, which may not be sufficiently represented by the information based on few species. The introduction of DNA based techniques as polymerase-chain reaction (PCR) or DNA-DNA checkerboard hybridization, improved the sensitivity and the accuracy of detection of multiple bacterial species. However, regardless of the improvement in the techniques, still a large part of the microbiome remained undetectable and the researchers tended to focus only on a limited number of targeted bacterial species.

The development of open-ended, DNA sequencing-based techniques for bacterial detection, gave the possibility to take a much more profound insight in the composition of the microbiome. In the last decade, the evolution from the traditional sequencing approach to the next generation sequencing methods resulted in an enlargement of the output information and in a significant cut in the costs of these technologies. Also new methods and programs for data analysis were employed. These new open-ended techniques plus modern statistical approaches have been applied to the oral and subgingival microbiome. These methods make it also possible to explore the relationship between the different bacterial communities. Thanks to the new amount of information, new taxa belonging to phyla *Bacteroidetes*, *Candidatus Saccharibacteria*, *Firmicutes*, *Proteobacteria*, *Spirochaetes* and *Synergistetes* have been associated to periodontitis (Griffen et al. 2012, Perez-Chaparro et al. 2014).

In this thesis, clearly the overlap of traditional culture techniques and more modern open-ended techniques is visible. While work was in progress, we could study the microbiology of periodontitis by culture up to next generation sequencing techniques.

### **Association between periodontitis and cardiovascular diseases**

In the late '80s, an epidemiological study from a Finnish group showed that a poor dental status was significantly associated with an increased risk for myocardial infarction (Mattila et al. 1989). The rationale of this study was the increased evidence that, in general, infections and inflammatory processes may increase the risk for acute cardiovascular disease events (CVD) in susceptible patients. This study was followed by a number of larger epidemiological investigations which, were later summarized in two meta-analyses. The results were that periodontitis patients <65 years have an increased relative risk of 1.4 for CVD and of 1.8 for stroke (Dietrich et al. 2013, Humphrey et al. 2008, Janket et al. 2003).

There are mainly two mechanisms proposed that are at the basis of a possible causative link between periodontitis and atherosclerosis and CVD. First, bacteria and/or their antigens can enter the systemic circulation, frequently per day, through the ulcerated epithelium of the periodontal pocket (Loos 2005, Schenkein & Loos 2013). This phenomenon is conceivable since the periodontal wound has been estimated to extent up to 39 cm<sup>2</sup> in patients with severe periodontitis (Nesse et al. 2008). This has been established by investigations that showed oral bacteria in the systemic blood circulation, even after routinely chewing or brushing maneuvers (Bahrani-Mougeot et al. 2008, Lockhart et al. 2008). *In vitro*, *P. gingivalis* has shown to be able to invade endothelial cells, induce platelet aggregation and activation of the coagulation (Brodala et al. 2005, Holmlund et al. 2009, Imamura et al. 2001). Furthermore, DNA of many bacterial species and periodontal pathogens have been found in specimens of atherosclerotic plaques (Elkaim et al. 2008, Figuero et al. 2011, Ott et al. 2006). An association between increased levels of antibodies against periodontal bacteria and increased risk of CVD has also been shown (Pussinen et al. 2007). Secondly, local periodontal inflammation can result in an increase of the systemic inflammatory burden. In the periodontal pocket of periodontitis patients, the subgingival biofilm and antigens in the periodontal tissues chronically challenge the local immune response of the host which in turn releases an amount of pro-inflammatory cytokines. It is conceivable to hypothesize that locally produced cytokines can “spill” in the systemic circulation (Loos 2005, Van Dyke & van Winkelhoff 2013). This hypothesis is corroborated by several investigations, which

showed that in periodontitis patients, there is, on average, a mild increase in blood of markers of inflammation as C-reactive protein (CRP), Interleukin-6 (IL-6), leukocytes and fibrinogen in comparison to patients without periodontitis (Buhlin et al. 2009, Loos 2005, Loos et al. 2000, Nakajima et al. 2010, Paraskevas et al. 2008).

The above described chronic pro-inflammatory status in periodontitis patients could represent the link not only to the reported increased risk of atherogenesis and CVD, but also to other predisposing conditions as increased endothelial dysfunction and dyslipidemia in periodontitis patients. Moreover, the pro-inflammatory state could predispose to a prothrombotic state, which is a tendency of the blood to coagulate and therefore a condition that could increase the risk of CVD.

### **Association between periodontitis and metabolic disorders**

Next to atherosclerosis and CVD, periodontitis has been associated with type 2 diabetes mellitus. For many decades periodontitis has been considered as a complication of deregulated hyperglycemia in diabetic subjects (Taylor et al. 2013). The glycation of proteins and lipids, due to excessive plasma levels of glucose, causes the formation of advanced glycated end-products (AGEs), which interact with many cellular receptors, leading to increased cellular oxidant stress and pro-inflammatory activity. These reactions are, in turn, responsible of the onset of multiple events (e.g impairment of leukocyte and fibroblastic activity, microvascular damage) which lead to a dysregulation of the host immune system and wound healing. Poor glycemic control has also been associated with higher progression rate of periodontal breakdown in comparison with patients with well-regulated glycemia (Bandyopadhyay et al. 2010).

However, since type 2 diabetes is a condition that is aggravated by inflammation, a possible two-way relationship between periodontitis and diabetes has also been hypothesized (Grossi & Genco 1998). The increased systemic level of pro-inflammatory cytokines in periodontitis, could contribute to pancreatic beta-cell dysfunction and insulin resistance through the impairment of intracellular insulin signaling (Goldberg 2009, Lalla & Papapanou 2011). This is supported by evidence, which shows that periodontitis patients have poorer glycemic control and a higher risk of diabetic complications than diabetics without periodontitis. Furthermore, there is evidence that periodontal treatment can positively contribute to the glycemic control in diabetic patients (Simpson et al. 2010, Taylor et al. 2013, Teeuw et al. 2010).

A similar mechanism has been proposed to explain the epidemiological association between periodontitis and metabolic syndrome, which is a condition which predisposes for CVD and type 2 diabetes (Nibali et al. 2013). Metabolic syndrome has been described as a cluster of risk factors which includes glucose regulation impairment, central obesity, hypertension, and dyslipidemia (Grundy 2008). The mechanism, which could be responsible for the onset of metabolic syndrome, is a systemic pro-inflammatory status. Chronic systemic inflammation can increase oxidative stress, which is an imbalance between cell production of oxidant and anti-oxidant molecular species. Oxidative stress has been associated with the impairment of many biological mechanisms, in particular to the anti-inflammatory effect of insulin (Bullon et al. 2009). A typical example is the increase of oxidative stress in obese individuals; white fat cells, in the presence of excessive calories intake, produce a higher amount of inflammatory cytokines and adipokines, which in turn increases the systemic pro-inflammatory state (Dandona et al. 2005). Following the same pattern, it has been hypothesized that also the systemic pro-inflammatory state originating from periodontal inflammation, may contribute to the increase of oxidative stress and metabolic disorders.

### **Perspective of the links between periodontitis and systemic conditions**

The above mentioned relationship between periodontitis and atherosclerotic/metabolic diseases does not support any causative link. However, there are several investigations which report that periodontal patients, either systemically healthy or with comorbidities, show after periodontal treatment a reduction in their levels of inflammatory markers, namely levels of CRP and leukocyte count, improvement in their glycemic control and improvement in their endothelial function and lipid profiles (Bokhari et al. 2012, D'Aiuto et al. 2013, Teeuw et al. 2010, Teeuw et al. 2014, Tonetti et al. 2007). These findings help to suggest a causative link between periodontitis and atherosclerotic and metabolic diseases. However the large variety in study protocols, selection criteria, treatment modalities, study population's characteristics and limited follow-up time (the majority of studies limit their follow-up to 3 to 6 months), make the current evidence still insufficient to fulfill the Bradford Hill criteria in order to draw definitive conclusions about the systemic effects of periodontal treatment and to claim causal relationship between periodontitis and atherosclerotic and metabolic diseases (Linden et al. 2013).

### **Aims of the thesis and thesis outline**

The first part of this PhD thesis focuses on the clinical, microbiological and systemic characteristics of periodontitis patients without comorbidities. In the second part, an intervention study focuses on the changes of those characteristics after treatment.

To study the link between periodontitis and cardiovascular diseases, first the relationship between periodontitis and a prothrombotic state as possible additional mechanism next to bacteremia and pro-inflammatory state was investigated (Chapter 2). To this aim a case-control study was set up using patients not reporting comorbidities and with or without periodontitis.

To explore more specifically a relationship of the microbiological burden in periodontitis and biomarkers of prothrombotic state, a study was initiated focusing on the immune response elicited by bacteria and their antigens in the circulation (Chapter 3). A way to measure the host response to the systemic bacteremia, is to measure the levels of immunoglobulins against specific periodontal pathogens in serum. Therefore we studied the association between the antibody levels against two well established periodontal pathogens (*A. actinomycetemcomitans* and *P. gingivalis*) and systemic levels of the biomarkers of prothrombotic state.

The second part of this thesis deals with the effects of basic periodontal treatment, with or without the adjunctive use of local antiseptics and/or systemic antibiotics, on clinical, microbiological and systemic characteristics in a cohort of periodontitis patients without systemic comorbidities.

In chapter 4, the clinical and microbiological effects of the adjunct therapies to basic periodontal therapy are investigated, in comparison to the basic periodontal therapy alone. These adjunctive treatments include local disinfection with an antiseptic (sodium hypochlorite 0.5%) with or without the use of systemic antibiotics (the combination of amoxicillin and metronidazole). In this chapter, microbiological analyses have been performed by culturing and focused on 7 bacterial species, which are traditionally associated with periodontitis.

In chapter 5 the systemic effects of the periodontal treatment were explored in relation to parameters of metabolic syndrome, a condition characterized by increased hypertension, increased visceral fat, hyperglycemia and dyslipidemia. This condition increases the risk to develop diabetes mellitus and CVD. Here it was aimed to investigate the change in the metabolic status, measured by 5 established markers of metabolic syndrome, in patients with periodontitis, but without comorbidities, after periodontal therapy.

An additional aim of this thesis was to study in depth the microbiological characteristics of periodontitis in the current intervention trial. In Chapter 6, baseline samples, taken from a subset of patients, were studied in order to compare the microbiological results between three different detection techniques. To this aim, two traditional established targeted techniques (culturing and PCR) and an open ended technique (454 DNA sequencing) were employed.

On the basis of the results in chapter 6, it was decided to study in a new subset of patients, by means of 454-pyrosequencing only, also the effect on the subgingival microbiome of the basic periodontal therapy, with or without the use of systemic antibiotics. Furthermore, the relation between the composition of the microbiome and the clinical outcomes of the two treatments was investigated (chapter 7).

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