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The Role of Immuno-Inflammatory Response in the Pathogenesis of Chronic Periodontitis and Development of Chair-Side Point of Care Diagnostics

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1. Introduction

Periodontitis is a chronic infection that results from the interaction of periodontopathogenic bacteria and host inflammatory and immune responses and is the most common bacterial infection worldwide. Estimates reveal that 10-15 % of adults have advanced periodontitis, and periodontal disease can contribute to widespread oral health dysfunction and enhanced susceptibility to other systemic diseases (Pussinen et al. 2007).

Bacterial biofilms are regarded to be the primary aetiological factor in the initiation of gingival inflammation and subsequent destruction of periodontal tissues (Offenbacher 1996) and three major specific pathogens have been repeatedly identified as etiologic agents, namely *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf) (Socransky et al. 1998). Although chronic exposure to bacteria and their products is a prerequisite for gingival inflammation and periodontal tissue destruction to occur, the major causative factor of soft- and hard- tissue breakdown associated with periodontitis is currently attributed to the host's immune-inflammatory response to bacterial challenge. Furthermore, the nature of the inflammatory response might determine the destructive character of the disease (Gemmell, Yamazaki, and Seymour 2002).

The theoretical manner in which periodontal disease progresses has long been a subject of debate. It is currently agreed that destructive periodontal disease progresses by means of asynchronous bursts of activity (Haffajee and Socransky 1986). According to this theory, periodontal tissue support is lost during short, acute episodes followed by prolonged periods of quiescence (Reddy, Palcanis, and Geurs 1997). This model implies that etiologic factors involved in periodontal tissue destruction would change according to the sequential occurrence of episodes of disease activity and quiescence or remission.

Determination of periodontal diagnosis and the extent and severity of periodontal tissue damage through standard periodontal assessment has traditionally been based on an array of clinical measurements, including probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), plaque index (PI) and radiographic findings. Though disease activity is generally associated to the loss of soft or hard tissue attachment to the tooth, recording of clinical attachment by periodontal probing at sequential examinations is the most common method to diagnose a progressive periodontal disease (Reddy, Palcanis, and Geurs 1997). However, clinical measurements provide information about past periodontal tissue destruction and do not elucidate the current state of the disease activity nor predict the future bone resorption (Armitage 2004). Thus, despite the value of these clinical methods, such techniques often result in inconsistent diagnoses, as well as an inability to reliably predict a patient's response to treatment (Offenbacher et al. 2007). A reason for the limited success in predicting the future course of disease in some individuals is that the clinical phenotype does not reflect the underlying biologic processes that occur at the biofilm-gingival interface (Offenbacher et al. 2007).

The biologic phenotype underlying chronic periodontitis, including the biofilm and the host response, tend to vary among individuals despite a similar clinical diagnostic category (Offenbacher et al. 2007). Consequently, disease screening should ideally be based on clinical determinations and the biologic phenotype (Page and Kornman 1997). Other associated factors include environmental exposures, as well as differences in genetic and possibly epigenetic composition (Page and Kornman 1997).

The biological changes underlying the transition process from gingival health to early inflammatory changes involve local increase in vascular permeability, edema and the recruitment and activation of polymorphonuclear neutrophils (PMN) (Delima and Van Dyke 2003). Acquired immune response becomes involved once antigen-presenting cells interact with immunocompetent cells, such as T and B lymphocytes, leading to the expansion of antibody-secreting plasma cells and the development of the chronic lesion (Gemmell and Seymour 2004). Bacterial-host interactions at the biofilm-periodontium interface trigger the synthesis of cytokines and other inflammatory mediators that promote the release of enzymes and bone-associated molecules that finally induce the alterations of the connective tissue metabolism and the destruction of the tooth supporting alveolar bone (Bhavsar, Guttman, and Finlay 2007; Graves 2008; Hourii-Haddad, Wilensky, and Shapira 2007).

In addition to local periodontal tissue involvement, chronic infection of the periodontium together with continuous up-regulation of pro-inflammatory responses and immune mediators may contribute to systemic sequel including diabetes, preterm delivery of low-weight birth babies, lung inflammation, arthritis and cardiovascular diseases (CVD). In fact, numerous case-control and cohort studies have demonstrated that periodontitis patients exert increased risk for CVD, acute myocardial infarction (AMI), peripheral arterial disease and CVD, relative to patients with healthy periodontium (Mattila, Pussinen, and Paju 2005; Chen et al. 2008; Mattila et al. 2000; Alfakry et al. 2011; Persson et al. 2003). Although the associations of periodontal diseases with CVD have been investigated in several clinical studies the pathogenic mechanisms and links between both diseases are not completely clarified (Bahekar et al. 2007; Buduneli et al. 2011).

A major challenge in clinical periodontics is to find a reliable molecular marker of periodontal support loss with high sensitivity, specificity and utility (Buduneli & Kinane 2011). Molecules derived from inflamed host tissue and pathogenic bacteria have the potential of being used as markers of periodontitis; however, molecular markers of bone resorption have advantages as they relate to specificity for bone, easy detection, pre-analytic stability and availability of sensitive and specific assays for detection (Forde et al. 2006). Up to now, at least 90 different components in gingival crevicular fluid (GCF) and oral fluids have been evaluated as possible biomarkers for diagnosis of periodontal disease and they can be divided into three major groups: (1) host derived enzymes and their inhibitors, (2) inflammatory mediators and host response modifiers, and (3) by-products of tissue breakdown, mainly of bone resorption (Lamster and Ahlo 2007).

Recently, the use of oral fluids such as GCF, whole saliva and oral rinse as a means of evaluating host-derived products, as well as exogenous components (for instance: oral microorganisms and microbial products), has been suggested as potential sources and diagnostic markers, respectively for disease susceptibility (Sahingur and Cohen 2004; Buduneli and Kinane 2011). In fact, as whole saliva represents a pooled sample with contributions from all periodontal sites, analysis of biomarkers in saliva may provide an overall assessment of disease status as opposed to site-specific GCF analysis. This review will analyze the mechanisms involved in the breakdown of periodontal supporting tissues during chronic periodontitis, with a special focus on the role of T cells, matrix metalloproteinases (MMPs) and the development of chair side point-of-care diagnostic aids applicable to monitor both, periodontal and systemic inflammation.

2. T cells and related cytokines

Nowadays, it has been clearly demonstrated that increases in receptor activator of nuclear factor-kappa B ligand (RANKL) mRNA and protein levels in periodontal tissues stimulate the differentiation of monocyte-macrophage precursor cells into osteoclasts and the maturation and survival of the osteoclasts, leading to alveolar bone loss (Hernandez et al. 2006; Ohyama et al. 2009; Nagasawa et al. 2007; Gaffen and Hajishengallis 2008; Crotti et al. 2003; Hofbauer and Heufelder 2001; Kawai et al. 2006; Vernal et al. 2004). In this context, during inflammatory response characteristic of periodontitis, proinflammatory cytokines, such as interleukin (IL)-1 β , IL-6, IL-17, and tumor necrosis factor (TNF)- α , can stimulate periodontal osteoblasts to express membrane-bound RANKL (Gaffen and Hajishengallis 2008; Acosta-Rodriguez et al. 2007; Harrington, Mangan, and Weaver 2006; Mosmann and Sad 1996; Graves 2008). In addition to osteoblasts, RANKL is expressed by a number of other cell types, mainly CD4⁺ T lymphocytes (Kawai et al. 2006).

CD4⁺ T lymphocytes represent one of the main components of the adaptive immune response and are the predominant cell type present in periodontitis gingival tissues (Hofbauer and Heufelder 2001; Kawai et al. 2006). After antigenic stimulation, naïve CD4⁺ T cells proliferate and may differentiate into distinct effector subsets, which have been classically divided on the basis of their cytokine production profiles into T helper (Th) 1 and Th2 cells (Mosmann et al. 1986). Th1 cells are characterized by the secretion of interferon (IFN)- γ , IL-2, IL-12, TNF- α and TNF- β , and are involved in the eradication of intracellular pathogens. Conversely, Th2 cells are characterized by secretion of IL-4, IL-5, IL-6, IL-9 and IL-13, which are potent activators of B cells, are involved in the elimination of extracellular

microorganisms and parasitic infections, and are also responsible for allergic disorders (Mosmann and Sad 1996; Mosmann and Coffman 1989).

More recently, two new subsets of CD4⁺ T lymphocytes have been characterized, the Th17 subset, which follows different polarizing conditions and displays different functional activities than Th1 and Th2 cells, and the regulatory T (Treg) cell subset with suppressor functions (Mosmann and Sad 1996). Activated human Th17 cells are phenotypically identified as CCR2⁺CCR5⁻ (Honma et al. 2007), whereas human memory CD4⁺ T cells producing IL-17 and expressing transcription factor related to orphan nuclear receptor C2 (RORC2) mRNA are CCR6⁺CCR4⁺ (Acosta-Rodriguez et al. 2007). Th17 cells secrete several pro-inflammatory cytokines such as IL-6, IL-17, IL-21, IL-22, IL-23, IL-26, TNF- α , and particularly RANKL (Liang et al. 2006; Harrington et al. 2005; Park et al. 2005).

The role of Th17 cells in host defence against pathogens is just emerging, particularly on their destructive potential in periodontal diseases. Increased levels of IL-17 were detected in GCF and in biopsy samples from periodontal lesions, both at the mRNA and protein levels, in patients with chronic periodontitis and these increased levels have been associated to CD4⁺ T cells (Takayanagi 2005; Vernal et al. 2005; Takahashi et al. 2005). Furthermore, RANKL was synthesized within periodontal lesions where IL-17 was produced by activated gingival T cells (Takahashi et al. 2005; Kramer and Gaffen 2007). These data are reinforced by the over-expression of RORC2 mRNA in active lesions from chronic periodontitis patients (Dezerega et al. 2010). Taken together, these data establish that Th17 cells represent the osteoclastogenic Th subset on CD4⁺ T lymphocytes, inducing osteoclastogenesis and bone resorption through synthesizing IL-17 and RANKL (Figure 1).

Diverse studies have analyzed the concentrations of RANKL and osteoprotegerin (OPG) in GCF of periodontitis patients and healthy subjects. In general, they show great variation from study to study, but the ratio of RANKL/OPG has a consistent tendency to increase from periodontal health to periodontitis and to decrease following non-surgical periodontal treatment (Bostanci et al. 2008, Buduneli et al. 2009). In a cross-sectional study, Bostanci *et al.* (2008) quantified the RANKL and OPG levels in GCF from 21 healthy subjects, 22 gingivitis, 28 chronic periodontitis (CP), 25 generalized aggressive periodontitis (GAgP) and 11 CP immunosuppressed patients, detecting that RANKL levels increased and OPG decreased in periodontitis patients compared with either gingivitis or healthy individuals, and concluded that RANKL/OPG ratio may predict disease occurrence (Bostanci et al. 2007). The same authors analyzed the GCF levels of TACE, an enzyme involved in the activation and secretion of RANKL from Th17 lymphocytes. They found that GCF TNF- α converting enzyme (TACE) levels were higher in periodontitis and TACE showed positive correlation with PD, CAL, and GCF RANKL concentration (Bostanci et al. 2008). In an intervention study (Buduneli et al. 2009), GCF levels of RANKL, OPG, and IL-17 were determined at baseline and also 4 weeks after completion of initial periodontal treatment in 10 smoker and 10 non-smoker patients with chronic periodontitis. The authors concluded that neither smoking nor periodontal inflammation seemed to influence GCF RANKL levels in systemically healthy patients with chronic periodontitis. Smoking and non-smoking patients with chronic periodontitis were affected similarly by the initial periodontal treatment with regard to GCF IL-17 and OPG concentrations.

On the other hand, Buduneli *et al.* (2008) selected 67 untreated CP and 44 maintenance patients and established RANKL and OPG salivary levels, demonstrating that RANKL and OPG may be affected by smoking and significant differences between treated *versus* untreated CP were found. CP patients (35 subjects) and 38 periodontally healthy subjects were analyzed by Sakellari *et al.* (2008). The GCF levels of RANKL increased in CP patients compared with healthy controls and these higher levels correlated with detection of *Treponema denticola* and *Porphyromonas gingivalis*, but not with clinical parameters (Sakellari, Menti, and Konstantinidis 2008). Arikan *et al.* (2011) evaluated RANKL, OPG, ICTP, and albumin levels in peri-implant sulcular fluid samples from 18 root-type implants with peri-implantitis in 12 patients and 21 clinically healthy implants in 16 other patients. The authors suggested that local levels of carboxyterminal telopeptide pyridinoline cross-links of type I collagen (ICTP) and OPG reflect an increased risk of alveolar bone loss around dental implants, and their local levels may help to distinguish diseased and healthy sites (Arikan, Buduneli, and Lappin 2011). Finally, Silva *et al.* (2008) performed a longitudinal follow-up of 56 patients affected of moderate to severe CP until determination of disease progression, detecting higher RANKL, IL-1 β levels and MMP-13 activity in active sites compared with inactive sites (Silva *et al.* 2008). Taken together, RANKL levels are promising as discloser of periodontal disease activity. Finally, carboxyterminal telopeptide pyridinoline cross-links of type I collagen (ICTP), released into the periodontal tissues as a consequence of MMP-mediated alveolar bone resorption has been suggested to predict future bone loss, to correlate with clinical parameters and putative periodontal pathogens, and also to reduce following periodontal therapy, representing a potentially valuable diagnostic marker for periodontal disease (Giannobile, Al-Shammari, and Sarment 2003).

3. Matrix metalloproteinases (MMPs): Destructive versus regulative roles

Periodontal tissue homeostasis depends on the balanced and regulated degradation of extracellular matrix (ECM) proteins. In addition, the molecular organization of extracellular matrix is known to profoundly influence cell behavior. An unbalance in favor of collagenous matrix degradation will result in the loss of periodontal supporting tissue, the hallmark of chronic periodontitis (Reynolds and Meikle 1997). MMPs enclose a family of genetically distinct but structurally related zinc-dependent proteolytic enzymes that can synergistically degrade almost all extracellular matrix and basement membrane components and regulate several cellular processes, including inflammatory responses (McQuibban *et al.* 2001; McQuibban *et al.* 2002; Overall, McQuibban, and Clark-Lewis 2002). The 23 MMPs expressed in humans are classified based on their primary structures and substrate specificities into different groups that include collagenases (MMP-1, -8, -13), gelatinases (MMP-9, -2), membrane-type MMPs (MT-MMPs, MMP-14, -15, -16, -17, -24, -25) and other MMPs (Folgueras *et al.* 2004).

MMPs share a basic structure composed of three domains, namely the pro-peptide, catalytic and the hemopexin-like domain; the latter is linked to the catalytic domain via a flexible hinge region. The proteolytic activity of MMPs is subjected to a complex regulation that involves three major steps (Kessenbrock, Plaks, and Werb 2010): 1) gene expression, 2) conversion of zymogen to active enzyme and 3) specific inhibitors. MMPs are initially synthesized as pro-enzymes which are enzymatically inactive because of the interaction between the cysteine residue of the prodomain with the zinc ion of the catalytic site, known as cysteine switch. Disruption of this interaction through proteolytic removal of the

prodomain or chemical modification results in enzyme activation. There are several proteinases that mediate MMP activation, including plasmin, furin and active MMPs that assemble in enzymatic amplifying loops. Once activated, the most important physiological inhibitors are tissue inhibitors of MMPs (TIMPs) -1, -2, -3 and -4 (Folgueras et al. 2004). Herein, the pathophysiological significance of increased MMP expression in periodontitis will rely ultimately on the presence of endogenous inhibitors and activating enzymes that will determine overall MMP activity (Sorsa, Mantyla et al. 2011; Buduneli and Kinane 2011).

MMPs, especially those with collagen-degrading properties, such as MMP-8, MMP-13 and MMP-9, have been recognized as the key proteases involved in destructive periodontal diseases and have widely been demonstrated in inflamed periodontal tissues and in oral fluids in association with supporting tissue loss by different analytic methods, including ELISA, immunofluorometric assays (IFMA), checkerboard method and immuno blots, (Folgueras et al. 2004; McQuibban et al. 2001; McQuibban et al. 2002; Overall, McQuibban, and Clark-Lewis 2002; Sorsa et al. 2006; Sorsa, Tjaderhane, and Salo 2004). All human MMPs are known to exist in multiple forms, i.e. latent pro-forms, active or activated forms, fragmented species, complexed species and cell-bound forms (Sorsa, Mantyla et al. 2011). The expression of different MMP isoforms in oral fluid samples can be analyzed with western immunoblotting, whereas a limitation of conventional MMP immunoassays used in periodontal research, such as ELISA, is that they do not differentiate these forms.

MMP-8 is mainly produced by neutrophils (PMN), but it can also be expressed by gingival fibroblasts, endothelial cells, epithelial cells, plasma cells, macrophages and bone cells (Heikkinen et al. 2010). MMP-8 is the major collagenolytic MMP in gingival tissue and oral fluids and elevated levels have been associated with the severity of periodontal inflammation and disease (Mantyla et al. 2006), whereas basal physiologic levels might be associated to tissue homeostasis and even to be protective against disease (Kuula et al. 2009). Among total collagenases in GCF, MMP-8 accounts for about 80%, whereas MMP-13, for up to 18% and MMP-1 is seldom detected (Golub et al. 2008).

MMP-13 has been identified in gingival sulcular epithelium, fibroblasts, macrophages, plasma cells and osteoblasts (Tervahartiala et al. 2000; Hernandez et al. 2006; Rydziel, Durant, and Canalis 2000). MMP-13 has been implicated in bone resorptive process, along with MMP-9 (Hill et al. 1995; Hill et al. 1994; Holliday et al. 1997). Total MMP-13 levels, as well as proenzyme (~60 kDa) and its active forms (~45-50 kDa), have been shown to increase in chronic periodontitis versus healthy sites in GCF in association with clinical periodontal parameters (Tervahartiala et al. 2000; Kiili et al. 2002; Ilgenli et al. 2006).

MMP-9 is the major gelatinase in oral fluids (Makela et al. 1994). As MMP-8, MMP-9 is present in granules of PMN and it is also expressed in a variety of other cell types, including resident periodontal cells, such as fibroblasts, keratinocytes and infiltrating leukocytes, like macrophages and plasma cells (Sorsa, Tjaderhane, and Salo 2004; Makela et al. 1994). Total MMP-9 levels and its active form have been demonstrated to significantly increase with periodontal inflammation in comparison to controls, composed of gingivitis and healthy subjects, and to drop along with inflammation after periodontal therapy (Makela et al. 1994; Bildt et al. 2008) (Figure 1).

Genetic variations can influence MMP transcription levels and protein synthesis. Despite the genetic background of periodontal diseases and the wide involvement of MMP-9, MMP-8 and MMP-13, MMP gene polymorphisms studied in different ethnic populations have not

been able to conclude specific associations with the susceptibility to develop periodontitis or disease severity. Similar allele and genotype frequencies have been demonstrated at the MMP-9 -1562 and -R+279Q polymorphic sites between periodontitis patients and healthy controls, despite the presence of significantly increased protein levels in serum and saliva of diseased subjects (Isaza-Guzman et al. ; Loo et al. 2011; de Souza et al. 2005; Chen et al. 2007; Gurkan et al. 2008). Nevertheless, the -1572T allele might be associated with a severe form of chronic periodontitis in men (Holla et al. 2006). Similarly, no differences in MMP-13- 77 A/G and -11 A /12 A polymorphic sites have been found for periodontitis patients (Pirhan et al. 2009), whereas no genetic studies are currently available for MMP-8.

Regarding, especially MMP-8, -9 and -13, it is noteworthy that clinical progression of periodontitis in active *versus* inactive sites and/or patients has been repeatedly demonstrated to be reflected as pathologically excessive elevation of either active MMP forms, i.e. conversion of latent pro-form to active form, or enzyme activity assessed by functional assays, i.e. total activated enzyme unbound to TIMPs, in GCF/peri-implant sulcular fluid (PISF), mouth-rinse and saliva samples collected from periodontitis/peri-implantitis sites and patients (Hernandez et al. 2010; Hernandez Rios et al. 2009; Sorsa, Mantyla et al. 2011). Regarding periodontitis/peri-implantitis progression in disease-active sites, pro-MMP-8, -9 and -13 have been demonstrated to be activated by independent and/or co-operative cascades involving other host proteinases (MMPs, serine proteases), reactive oxygen species and/or microbial proteases (Buduneli et al. 2011; Hernandez et al. 2010; Hernandez Rios et al. 2009). GCF collagenase activity and MMP-8 activation are also found to correlate with the levels of type I collagen breakdown fragments overcoming the protective shield provided by TIMP-1 (Reinhardt et al. 2010; Sorsa, Mantyla et al. 2011). Similarly, MMP-13 activity and ICTP have shown to increase in active sites compared with inactive sites from progressive periodontitis patients or healthy subjects (Hernandez Rios et al. 2009).

Clinical trials testing subantimicrobial dose doxycycline (SDD, synthetic FDA-approved MMP-inhibitor) medication have repeatedly reported an association between improvement of clinical parameters and reduction of GCF and serum MMP-8, -13 and -9 activation and levels (Reinhardt et al. 2010, Sorsa et al. 2011). It is possible to monitor the effect of periodontal treatment and adjunctive SDD medication by point-of-care MMP-8 immunoassays (Sorsa, Tervahartiala et al. 2011).

MMPs can also act by regulating many other MMP activation cascades. This later role could even be more significant in periodontal tissue destruction than direct collagenolytic activity, in a way that a subtle change in regulating MMPs might result in widespread MMP activation and consequent tissue destruction (Folgueras et al. 2004; Hernandez et al. 2010). MMP-14 activates the collagenases MMP-8 and MMP-13 *in vitro* (Holopainen et al. 2003; Folgueras et al. 2004; Han et al. 2007; Dreier et al. 2004; Knauper et al. 1996). Several studies using experimental models associate MMP-14, -13, -9 and -2 over expression with bone resorption, and the inhibition of bone loss by the addition of an MMP inhibitor (de Aquino et al. 2009; Rifas and Arackal 2003; Garlet et al. 2006; Cesar Neto et al. 2004; Trombone et al. 2009). *In vitro* studies have revealed that MMP-13 might initiate bone resorption by generating collagen fragments that can activate osteoclasts (Holliday et al. 1997) and proMMP-9 (Knauper et al. 1997). Active MMP-9 in turn, further digests denatured collagen derived from MMP-13 activity (Hill et al. 1995), is thought to act over preosteoclast recruitment to sites for osteoclast differentiation and bone resorption, and activates

proMMP-13 and proMMP-2. On the other hand, proMMP-13 can also be activated by active MMP-14, MMP-2 and MMP-13 *in vitro* (Knauper et al. 1996). Nevertheless, whether these enzymes proteolytically interact *in vivo* is not clear, yet.

Adding of recombinant MMP-13 to gingival tissue from chronic periodontitis patients has been reported to result in elevated proMMP-9 and proMMP-2 activation rates, but only the former was significant. Furthermore, addition of MMP-13 specific synthetic inhibitor CL-82198 prevented proMMP-9 activation (Hernandez Rios et al. 2009). These results suggest an activation cascade involving MMP-13, MMP-9 and possibly MMP-2 in periodontitis progression. Higher MMP-14 levels have also been found in gingival tissue from periodontitis subjects compared to healthy gingiva (Oyarzun et al. 2010) and soluble forms of MMP-14 have been described in periodontitis GCF (Tervahartiala et al. 2000), showing a trend to be higher in active sites in comparison to inactive ones in patients with progressive periodontitis (Hernandez et al. 2010). Moreover, a positive correlation between active MMP-14 and active MMP-13 in periodontitis GCF was found, suggesting proteolytic activation to occur *in vivo* (Hernández et al. 2011). Thus, this novel proteolytic cascade could perpetuate periodontal soft and hard tissue destruction in a feed forward manner. Conversely, MMP-14 has been inversely correlated to MMP-8 in GCF from active sites in progressive periodontitis, and thus this proteolytic activation mechanism might be of minor importance in disease progression (Hernandez et al. 2010; Hernandez, Dutzan et al. 2011).

MMPs can also regulate many biological processes through limited proteolysis of matrix and non-matrix bioactive molecules, such as immune-inflammatory response and wound healing, among others, through either 1) hydrolysis of extracellular matrix to allow cell migration, 2) cleavage of binding proteins and releasing of soluble bioactive molecules from extracellular matrix reservoir or cell compartment and 3) by processing bioactive molecules, modifying their biological activity (Korpi et al. 2009; Lin et al. 2008; Tester et al. 2007; Van Den Steen et al. 2003; Van Lint et al. 2005; Gutierrez-Fernandez et al. 2007; Hernandez et al. 2011).

It has been recently reported that *Porphyromonas gingivalis*-induced experimental periodontitis in MMP-8 knock-out mouse model resulted in a more severe disease phenotype and reduced levels of mouse lipopolysaccharide (LPS)-induced CXC chemokine (LIX/CXCL5) than their wild type counterparts (Kuula et al. 2009; Hernandez et al. 2011). LIX/CXCL5 and its human homologue, granulocyte chemotactic protein-2 (GCP-2/CXCL6) have been proposed to regulate neutrophil influx to periodontal tissues at the oral interface, where they represent the first line of defense against periodontal pathogens (Kebschull et al. 2009; Hernandez et al. 2011). Accordingly, several studies support a role for MMP-8 in PMN trafficking in different inflammation models. LIX/CXCL5 levels have also shown to diminish in other MMP-8 knock-out mouse models (Gutierrez-Fernandez et al. 2007; Nilsson, Jonsson, and Dabrosin 2009), such as TNF-induced lethal hepatitis model (Van Lint et al. 2005), and reduced levels of transforming growth factor (TGF)- β 1 in MMP-8^{-/-} mice have been associated with PMN impaired infiltration and wound healing (Gutierrez-Fernandez et al. 2007). All these potential regulatory mechanisms together or alone may lead to an impaired PMN influx to the sites of inflammation in MMP-8 deficient mice and alter disease expression. Similarly, MMP-9 activity was shown to down regulate TGF- β 1 protein levels in breast cancer cells exposed to tamoxifen (Nilsson, Jonsson, and Dabrosin 2009; Balbin et al. 2003). In addition, recent evidence supports that MMP-13 might also influence soluble protein levels from RANKL/OPG axis (Nannuru et al. 2010).

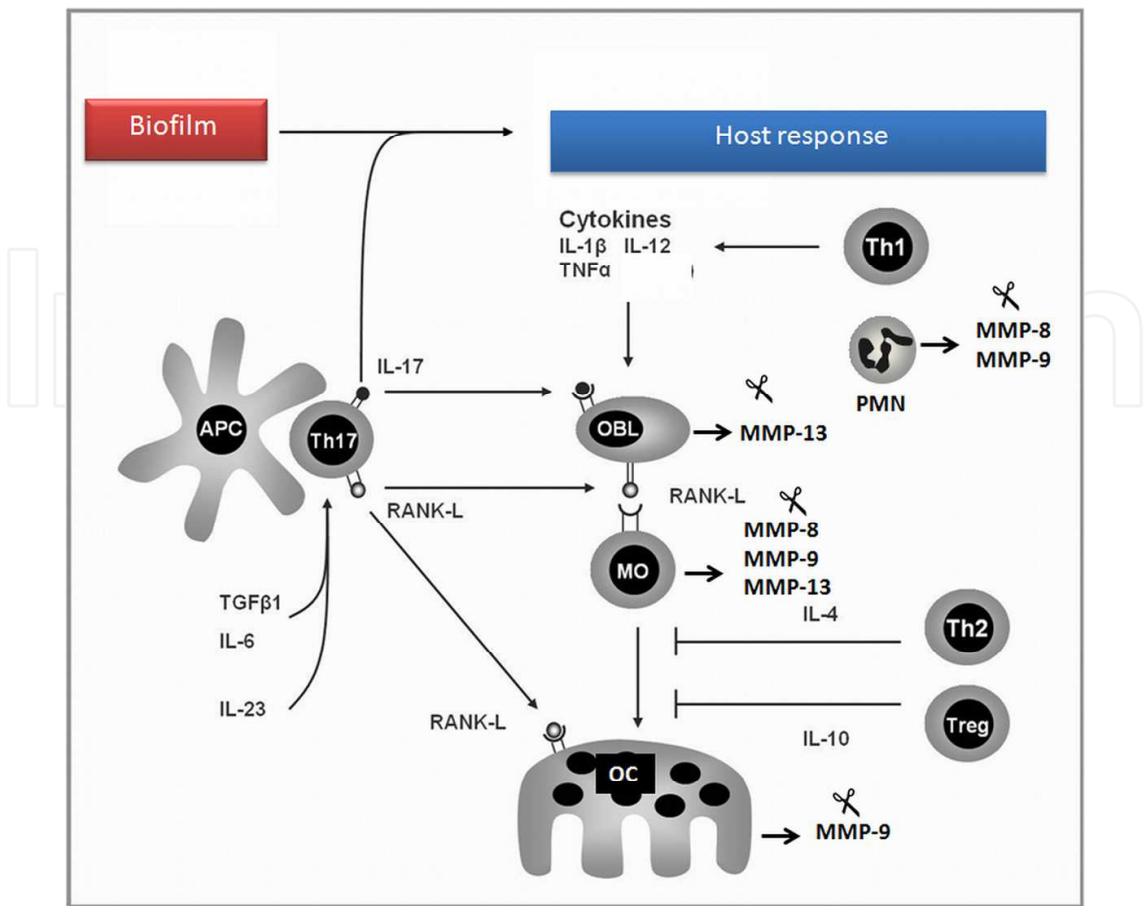


Fig. 1. The host response during periodontitis with emphasis on T cells and matrix metalloproteinases (MMPs) in periodontal tissue breakdown. As result of chronic stimuli from periodontopathogenic biofilm an immune-inflammatory response is established in periodontal tissues. The major features are the synthesis of inflammatory cytokines, such as IL-1 β , IL-12, TNF- α and pathologically elevated matrix metalloproteinases (MMPs) synthesis and activation, specially MMP-8, MMP-9, and MMP-13. These MMPs degrade extracellular matrix, particularly collagen I, and they also influence periodontal immune-inflammatory response through limited proteolysis. Th17 cells may be activated as consequence of antigen presentation and contribute to bone destruction by secreting IL-17 and RANKL. IL-17 increases the inflammatory response and induces RANKL expression by osteoblastic cells. RANKL induces an increment in the osteoclast differentiation/maturation and alveolar bone resorption. MMP-13 produced by osteoblasts and MMP-9 from osteoclasts participate in the degradation of organic bone matrix. IL: interleukin; MMP: matrix metalloproteinase; TNF: tumor necrosis factor; TGF: transforming growth factor; OC: Osteoclast; APC: Antigen presenting cell; PMN: neutrophil; MO: macrophage; OBL: osteoblast.

4. Oral fluid chair-side point-of-care technologies and their systemic applications

Oral fluids [GCF, saliva, mouth-rinse, PISF] contain various molecular mediators often called biomarkers that reflect several physiological and pathological conditions. Qualitative and quantitative changes in the oral fluid biomarkers have been found to exert significance in the adjunctive diagnostics and treatment of various oral and systemic disorders.

Periodontal diseases are reflected in oral fluids as elevated levels of host cell-derived tissue destructive proteolytic enzymes, i.e. MMP-8, -9 and -13, neutrophil elastase, α_2 -macroglobulin, oxygen radical producer myeloperoxidase (MPO), pro-inflammatory mediators, (C Reactive Protein (CRP), IL-1 β , TNF- α , macrophage inflammatory protein -1) and bone remodeling markers (alkaline phosphatase, ICTP, RANKL, osteoprotegerin and osteocalcin). Among these periodontitis-related oral fluid biomarkers, especially MMP-8, -9 and -13, as well as MPO are potential candidates for chair-side point-of-care oral fluid assays (Hernandez et al. 2010; Leppilahti et al. 2011; Sorsa, Tervahartiala et al. 2011). Low GCF volume and low biomarker levels in all oral fluids are characteristic during periodontal health, but the biomarker contents increase along with the severity of periodontal inflammation (Pussinen et al. 2007).

GCF, salivary and mouth-rinse biomarker analysis can provide adjunctive information for health care professionals alongside traditional oral clinical examination whether periodontal disease is present, whether treatment or medication is required or if the treatment or medication has been effective. GCF represents site specific analysis of studied biomarkers and volume. However, molecular analysis of GCF elution can be time consuming while most of the GCF analytic assays are laboratory based and usually cannot be performed in a chair-side manner. These procedures, as well as GCF sampling, are technically demanding and the GCF volume can be very small (1-5 μ l). Despite these apparent diagnostic and technical disadvantages, GCF is still considered as a candidate potential oral fluid for the development of adjunctive non-invasive chair-side point of-care diagnostic technology (Sorsa et al. 1999; Sorsa et al. 2006; Sorsa, Tjaderhane, and Salo 2004; Mantyla et al. 2006; Mantyla et al. 2003; Munjal et al. 2007), especially because tissue destructive MMPs and their bioactive regulators can conveniently be measured by distinct catalytic and non-catalytic immunoassays from GCF (Sorsa et al. 2010).

In relation to GCF, collection of salivary and mouth-rinse samples is more convenient, practical, rapid and non-invasive and requires neither professional stuff nor specific materials. It could even be carried out by patients themselves. Saliva and mouth-rinse represent a pooled sample from all periodontal sites providing an overall assessment of periodontal disease and health at subject level. Whole saliva can be affected by molecular constituents and cellular remnants from other oral niches, as well as systemic conditions (Buduneli et al. 2011; Buduneli and Kinane 2011) which should be considered when it is used for diagnostics.

MMP-8 or collagenase-2/neutrophil-collagenase is the major type of interstitial collagenase present in human periodontitis-affected gingival tissue, GCF, PISF, saliva and mouth-rinse samples (Sorsa et al. 2006). Antibodies applied in the immunoassays for the detection of MMPs and their regulators affect the measurement outcome (Leppilahti et al. 2011; Gursoy et al. 2010; Sorsa et al. 2010; Sorsa, Mantyla et al. 2011). Nevertheless, especially MMP-8 immunoassays and activity assays targeting PMN-type MMP-8 isoenzyme species in oral fluids have been found to be useful to differentiate periodontitis/peri-implantitis and gingivitis sites/patients as well as healthy sites/subjects (Mantyla et al. 2006; Mantyla et al. 2003; Hernandez et al. 2010; Sorsa et al. 2010; Sorsa, Mantyla et al. 2011). Although periodontal clinical examination is necessary and cannot be substituted by any other means in periodontal diagnostics, biomarker testing could give relevant clinical adjunctive information about the individual's host response levels, although there is no known normal range at present.

Selective antibodies for detection of active MMP-8 in oral fluids have been utilized as adjunctive diagnostic point-of-care/chair-side tests identifying sites susceptible for periodontitis progression and periodontitis affected patients (Leppilahti et al. 2011; Mantyla et al. 2006; Sorsa, Mantyla et al. 2011; Sorsa, Tervahartiala et al. 2011). We have recently investigated levels of GCF MMP-8 with two different chair-side (dentoAnalyzer by dentognostics GmbH and MMP-8 specific chair-side dip-stick test) and two laboratory methods (immunofluorometric assay, IFMA, and commercial ELISA) (Sorsa et al. 2010). IFMA, dentoAnalyzer and MMP-8 specific chair-side dip-stick test results were well in line. Results obtained with MMP-8 commercial ELISA kit were not in line with recordings by other methods. Both IFMA and dentoAnalyzer device detected the GCF samples' MMP-8 levels with equal reliability. The chair-side dip stick test results were in line with results with these two other methods but the capability of the dip-stick test to differentiate the sample levels were rougher. The chair-side dip-stick test detected especially the sites with high MMP-8 levels.

The differences between dentoAnalyzer, IFMA, dipstick and commercial ELISA MMP-8 analysis of GCF levels can be, at least in part, explained by the evidently different specificities and sensitivities between antibodies used in these assays. DentoAnalyzer, IFMA and dipstick assays use same antibody (Hanemaaijer et al. 1997). Regarding serum and plasma MMP-8 determinations by using both IFMA and commercial ELISA, significantly higher serum MMP-8 values were recorded relative to plasma, and the differences were most notable with high serum MMP-8 concentrations as measured using IFMA (Tuomainen et al. 2008; Emingil et al. 2008). The antibody used in dentoAnalyzer, IFMA and dip-stick exerts high sensitivity to both PMN- and fibroblast-type MMP-8 isotypes and especially their active forms (Hanemaaijer et al. 1997; Sorsa et al. 1999).

Although several studies have demonstrated the central role of MMP-8 in periodontitis, it has not been shown that it is predictive of disease progression, i.e. that the increased MMP-8 concentration in GCF would precede the occurrence of attachment loss (AL). This problem arises from the nature of periodontitis and from the accuracy of diagnosing a site as progressing with clinical or radiological methods. Disease progression is regarded to be mostly episodic, occurs only infrequently and is slow in most chronic periodontitis patients. During a study period, it is likely that only a small number of sites with AL can be confirmed. Additionally, only a small group of periodontitis patients manifest multiple progressing sites (Chambers et al. 1991; Mantyla et al. 2006). In our previous study, we could not make a conclusion about the predictive value of MMP-8 testing (Mantyla et al. 2006). However, we concluded that repeatedly elevated GCF MMP-8 levels indicate the sites at risk of periodontal AL and that testing of MMP-8 site specifically from GCF is a valuable diagnostic aid which supplements the traditional methods especially from selected sites in the maintenance phase of periodontitis patients who are at continuous risk for periodontitis recurrence. Periodontitis patients' GCF MMP-8 levels decrease after conventional periodontitis hygiene phase treatment. However, the levels remain higher than in gingivitis patients' or in periodontally healthy subjects' GCF (Mantyla et al. 2003). This is valid also in periodontitis patients' shallow sites, as well as sites with no attachment loss, and tells about the elevated basic host response of periodontitis subjects (Mantyla et al. 2006; Mantyla et al. 2003) (Figure 2).

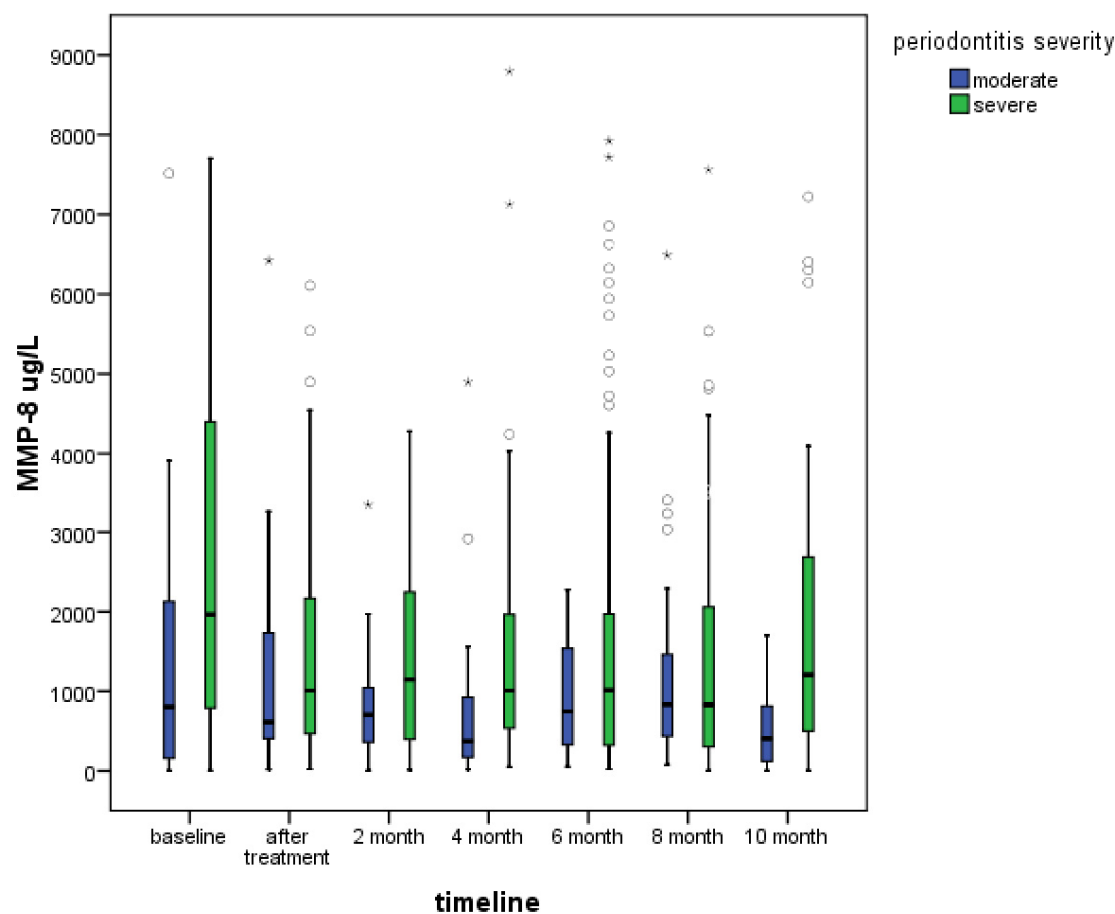


Fig. 2. **MMP-8 levels in gingival crevicular fluid from subjects with moderate and severe chronic periodontitis.** The box plot shows MMP-8 IFMA levels ($\mu\text{g/l}$) from patients suffering of moderate chronic periodontitis (4 patients, 34 sites) and severe chronic periodontitis (10 patients, 81 sites). Same sites from each patient were analyzed before any periodontitis treatment (baseline), one month after treatment (scaling and root planing), and with two-month interval during the maintenance phase (2-10 month). From each patient both shallow and deep sites at baseline were included into analyzed sites. The figure shows that GCF MMP-8 levels decrease in both groups after treatment but remain at comparatively high levels in severe periodontitis at each point in time indicating the higher level of host response. Box plots also show the wide ranges of MMP-8 levels, which are typical for biomarkers, and ranges become wider with the severity of the disease.

Based on other recent findings the salivary and oral rinse chair-side sample analysis of MMP-8 can be clinically useful in rough screening to identify individuals with periodontitis or to analyze the individual level of host response. Simultaneous analysis of MMP-8 and TIMP-1 could be beneficial (Leppilähti et al. 2011). The oral fluid testing could give valuable additional information about the control of inflammation which today is based on the clinical findings, that is shallower or eliminated periodontal pockets and less bleeding on probing. Salivary and oral rinse sample analysis may be useful in defining the optimal period between periodontal maintenance visits after active periodontal treatment. During the active treatment phase it could be possible to monitor the decrease of salivary and oral rinse MMP-8 levels; at the end of the active treatment phase it would show the individual optimal biomarker level to keep the host

response in control. At best the biomarker level could be monitored by screening home self-test to indicate when the cut-off for possibly unsafe biomarker level is reached. Also the effect of MMP-8 inhibiting SDD medication could be monitored by analyzing the salivary and oral rinse MMP-8 levels to find out when a possible break in medication would be possible or when the medication should be taken again (Golub et al. 2008; Reinhardt et al. 2010). Screening type testing from saliva or oral rinse would also be applicable for differentiation of the borderline between gingivitis and periodontitis, because gingivitis patients' MMP-8 levels are shown to be significantly lower than periodontitis patients' levels (Mantyla et al. 2003). Thus, the levels repeatedly approaching to those of periodontitis could be used to indicate patients at risk.

The point-of-care MMP-8 immuno-technologies from oral fluids and serum/plasma can be well adapted for monitoring of systemic inflammation (Tuomainen et al. 2007; Buduneli and Kinane 2011). In this regard, the elevation of serum and plasma MMP-8 has been associated with AMI, CVD and sepsis (Buduneli et al. 2011; Lauhio et al. 2011; Sorsa, Tervahartiala et al. 2011). Furthermore, a recent study on salivary MMP-8 assessed by Western immunoblot analysis in patients with or without acute myocardial infarction revealed that elevated salivary MMP-8 activation is associated with AMI (Buduneli et al. 2011). Moreover, elevated serum MMP-8 levels have recently been demonstrated to be associated with total outcome in a multicentre and prospective cohort study in sepsis; serum MMP-8 levels were significantly higher among non-survivors than among survivors. Thus elevated oral fluid and serum MMP-8 can be considered as a potential risk factor for systemic diseases such as, but not limited to, CVD, sepsis, diabetes, stroke, arthritis and pulmonary diseases (Kardesler et al. 2010; Biyikoglu et al. 2009; Buduneli et al. 2011; Ozcaka et al. 2011). In addition, oral MMP-8 activation as a consequence of AMI may contribute to progression of periodontitis and/or oral discomfort. However, oral fluid point-of-care diagnostics contains many potential confounders. The various medications together with systemic diseases (mental disorders / psychiatric diseases, nephritic syndrome) can affect the levels of potential periodontitis and CVD biomarkers in saliva and serum/plasma (Alfakry et al. 2011; Sorsa, Tervahartiala et al. 2011; Buduneli et al. 2011). Accordingly, the beneficial and reducing effects of lymecycline (a tetracycline-derivate) on serum MMP-8 levels could be monitored by MMP-8 immunoassay in reactive arthritis (Lauhio et al. 2011). In fact, serum MMP-8 levels may reflect defensive molecular processes in certain malignancies (Korpi et al. 2008) and also HIV-infection has been found to affect the level of MMPs and their regulators in oral fluid and serum/plasma (Mellanen et al. 2006).

5. Concluding remarks

Periodontal tissue destruction derived from chronic periodontitis occurs as consequence to the activation of immune-inflammatory response of the host to bacterial challenge. Major events comprise pro-inflammatory cytokine production and collagenolytic MMPs, such as MMP-8, MMP-13 and MMP-9 leading to soft periodontal tissue breakdown. Activation of Th17 subset of T lymphocytes will induce the synthesis of IL-17 and RANKL. These cytokines along with bone MMPs, will lead ultimately to osteoclastogenesis and alveolar bone resorption. Qualitative and/or quantitative changes in oral fluid biomarkers might be useful as adjunctive diagnostics and treatment of periodontitis. Among them, it appears that MMP-8 point-of-care diagnostics exert a huge potential in oral diagnostics and forms a diagnostic link from oral cavity to systemic inflammatory conditions.

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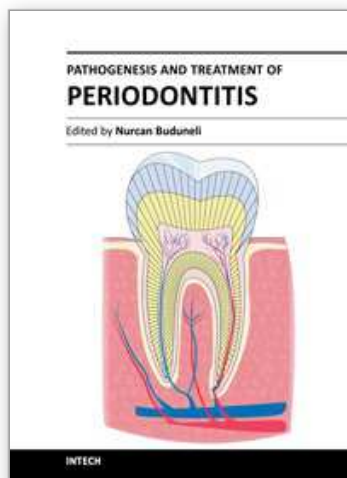
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