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IDENTIFICATION OF COLLAGENOLYTIC MMP NETWORKS AS POTENTIAL BIOMARKERS IN PROGRESSIVE CHRONIC PERIODONTITIS SUBJECTS AND MMP-8 NULL ALLELE MODEL

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Academic Dissertation

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ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
APMA	Aminophenylmercuric acetate
au/mL	Arbitrary units/mL
BOP	Bleeding on probing
CAL	Clinical attachment loss
CSF	Colony stimulating factor
CXCR	CXC chemokine receptor
FP	Fluorescent product
GCF	Gingival crevicular fluid
GCP-2/CXCL6	Human granulocyte chemotactic protein-2 /CXC chemokine-6
HE	Hematoxylin and eosin
HOCl	Hypochlorous acid
ICTP	Carboxyterminal telopeptide of type I collagen
IL	Interleukin
IQR	Interquartile range
KO	Knock out
LIX/CXCL5	Lipopolysaccharide-induced CXC chemokine-5
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant proteins
M-CSF	Macrophage colony-stimulating factor
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
OPG	Osteoprotegerin
P. gingivalis	*Porphyromonas gingivalis*
PD	Probing depth
PA	Plaque accumulation
PMN	Polymorphonuclear neutrophil
RANKL	Receptor activator of NF-κB ligand
ROS	Reactive oxygen species
SDD	Submicrobial dose doxycycline
SDF-1/CXCL12	Stromal-derived factor-1/CXC chemokine-12
SRP	Scaling and root planning
T. forsythia	*Tannerella forsythia*
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of matrix metalloproteinases
TNF	Tumor necrosis factor
WT	Wild type

ABSTRACT

Chronic periodontitis results from a complex aetiology, including the formation of a subgingival biofilm and the elicitation of the host's immune and inflammatory response. The hallmark of chronic periodontitis is alveolar bone loss and soft periodontal tissue destruction. Evidence supports that periodontitis progresses in dynamic states of exacerbation and remission or quiescence. The major clinical approach to identify disease progression is the tolerance method, based on sequential probing.

Collagen degradation is one of the key events in periodontal destructive lesions. Matrix metalloproteinase (MMP)-8 and MMP-13 are the primary collagenolytic MMPs that are associated with the severity of periodontal inflammation and disease, either by a direct breakdown of the collagenised matrix or by the processing of non-matrix bioactive substrates. Despite the numerous host mediators that have been proposed as potential biomarkers for chronic periodontitis, they reflect inflammation rather than the loss of periodontal attachment. The aim of the present study was to determine the key molecular MMP-8 and -13 interactions in gingival crevicular fluid (GCF) and gingival tissue from progressive periodontitis lesions and MMP-8 null allele mouse model.

In study (I), GCF and gingival biopsies from active and inactive sites of chronic periodontitis patients, which were determined clinically by the tolerance method, and healthy GCF were analysed for MMP-13 and tissue inhibitor of matrix metalloproteinases (TIMP)-1. Chronic periodontitis was characterised by increased MMP-13 levels and the active sites showed a tendency of decreased TIMP-1 levels associated with increments of MMP-13 and total protein

concentration compared to inactive sites. In study (II), we investigated whether MMP-13 activity was associated with TIMP-1, bone collagen breakdown through ICTP levels, as well as the activation rate of MMP-9 in destructive lesions. The active sites demonstrated increased GCF ICTP levels as well as lowered TIMP-1 detection along with elevated MMP-13 activity. MMP-9 activation rate was enhanced by MMP-13 in diseased gingival tissue. In study (III), we analysed the potential association between the levels, molecular forms, isoenzyme distribution and degree of activation of MMP-8, MMP-14, MPO and the inhibitor TIMP-1 in GCF from periodontitis progressive patients at baseline and after periodontal therapy. A positive correlation was found for MPO/MMP-8 and their levels associated with progression episodes and treatment response. Because MMP-8 is activated by hypochlorous acid *in vitro*, our results suggested an interaction between the MPO oxidative pathway and MMP-8 activation in GCF. Finally, in study (IV), on the basis of the previous finding that MMP-8-deficient mice showed impaired neutrophil responses and severe alveolar bone loss, we aimed to characterise the detection patterns of LIX/CXCL5, SDF-1/CXCL12 and RANKL in *P. gingivalis*-induced experimental periodontitis and in the MMP-$8^{-/-}$ murine model. The detection of neutrophil-chemoattractant LIX/CXCL5 was restricted to the oral-periodontal interface and its levels were reduced in infected MMP-8 null mice vs. wild type mice, whereas the detection of SDF-1/CXCL12 and RANKL in periodontal tissues increased in experimentally-induced periodontitis, irrespectively from the genotype. Accordingly, MMP-8 might regulate LIX/CXCL5 levels by undetermined mechanisms, and SDF-1/CXCL12 and RANKL might promote the development and/or progression of periodontitis.

1. INTRODUCTION

Periodontal diseases result from the interplay of diverse aetiologic factors, including the formation of a complex biofilm in the subgingival microenvironment, social and behaviour modulations, genetic or epigenetic traits and the host's immune and inflammatory response (Haffajee and Socransky, 1994).

The hallmark of chronic periodontitis is alveolar bone loss and soft periodontal tissue destruction (Vernal et al., 2004). Evidence supports that periodontitis progresses in dynamic states of exacerbation and remission (Jeffcoat and Reddy, 1991). The most common method used to diagnose attachment loss and, thereby, the disease progression, is to record the clinical attachment at sequential examinations by periodontal probing (Reddy et al., 1997). Periodontal supporting tissue homeostasis depends on extracellular proteolysis and remodelling, involving a tight balance among protease activities and inhibitors. Unhampered proteolysis results in collagenous matrix breakdown and the loss of periodontal supporting tissue. Pathologically enhanced expression and activation of host matrix metalloproteinases (MMPs) over the protective shield provided by their main tissue inhibitors (TIMPs) is a well-characterised feature in chronic periodontitis (Sorsa et al., 2006). Although MMPs have been assumed to primarily play a matrix degradative role, a broader substrate degradome has shown proteolytic susceptibility to MMPs, including cytokines, chemokines, other MMPs and apoptotic signals. As a result, MMPs can also modulate immune and inflammatory responses, among other cellular processes (Folgueras et al., 2004; Sorsa et al., 2004; Sorsa et al., 2006).

Collagen degradation is regarded as one of the key events in periodontal destructive lesions (Golub et al., 1997; Kiili et al., 2002). The major collagenolytic MMPs associated with the severity of periodontal inflammation and disease are collagenase-2 (MMP-8) and collagenase-3 (MMP-13); whereas collagenase-1 (MMP-1) is primarily related to physiological periodontal tissue turnover (Kiili et al., 2002; Makela et al., 1994; Sorsa et al., 1988; Sorsa et al., 2004; Sorsa et al., 2006; Tervahartiala et al., 2000; Uitto et al., 2003). Despite the fact that many potential biomarkers have been proposed in chronic periodontitis, most of them demonstrate limited usefulness because they reflect periodontal inflammation rather than disease progression (Loos and Tjoa, 2005). While numerous cross-sectional studies have shown evidence that MMP levels parallel the severity of periodontal inflammation, much less data are available from active periodontal destructive lesions assessed by longitudinal studies. Pathologically excessive collagenase levels and activities in progressive periodontitis-affected tissue and oral fluids might provide a proteolytic signature of the underlying biological phenotype that might contribute to the development of chair-side point of care diagnostics, in order to improve clinical follow-ups, treatment decisions and future drug development (Leppilahti et al., 2011; Mantyla et al., 2003; Mantyla et al., 2006; Sorsa et al., 2004; Sorsa et al., 2006). The purpose of this study was to find the key molecular collagenolytic MMP-interactions/cascades in gingival crevicular fluid (GCF) and gingival tissue from destructive periodontitis lesions and MMP-8 null allele mouse model that might be suitable for potential point-of-care/chair-side diagnostic development for chronic periodontitis.

2. REVIEW OF THE LITERATURE

2.1 General aspects of periodontal health and disease

2.11 Healthy periodontium

The periodontium is defined as the tissues supporting and surrounding the tooth and corresponds to the root cementum, periodontal ligament, alveolar bone and gingiva. The gingiva part that faces the tooth is known as the dentogingival junction. The junctional epithelium is crucial among the components of the dentogingival junction because it seals off the rest of the periodontal tissues from the oral environment and represents the first line of defence, while structural alterations represent the first step towards the development of periodontal disease. The epithelial attachment corresponds to the layer of the junctional epithelium that faces the tooth, providing the actual attachment among them. Even in clinically normal circumstances, the connective tissue supporting the junctional epithelium shows an inflammatory cell infiltrate composed of neutrophils and T lymphocytes that is regarded to influence the overlying epithelial phenotype (Nanci and Bosshardt, 2006) (Figure 1.).

2.12 Periodontal diseases

Periodontal diseases usually refer to the common inflammatory disorders of gingivitis and periodontitis caused by pathogenic microbiota harvested in the biofilm or dental plaque that forms adjacent to the teeth. Gingivitis is an inflammatory condition of the gingiva. Gingivitis can be modified by several factors, such as smoking and hormonal changes, and

it clinically displays the typical cardinal signs of inflammation (Kinane, 2001). Periodontitis follows gingivitis and is influenced by the host's immune and inflammatory responses. Periodontitis involves the irreversible destruction of the supporting tissues, including the periodontal ligament, alveolar bone and soft tissues; if left untreated, it often leads to tooth loss (Kinane, 2001; Socransky and Haffajee, 2005) (Figure 1.). However, not all gingivitis progresses towards periodontitis; in addition, periodontitis does not affect all teeth evenly, but it does have both a subject and site predilection. Furthermore, only relatively few sites undergo extensive periodontal destruction within a given observation period.

Chronic periodontitis is the most common form of periodontitis; it occurs primarily in adults, and it is generally a slowly progressing disease that is associated with the presence of subgingival calculus. Overall, periodontal disease progression can be considered a continuous process that undergoes periods of exacerbation (Kinane, 2001).

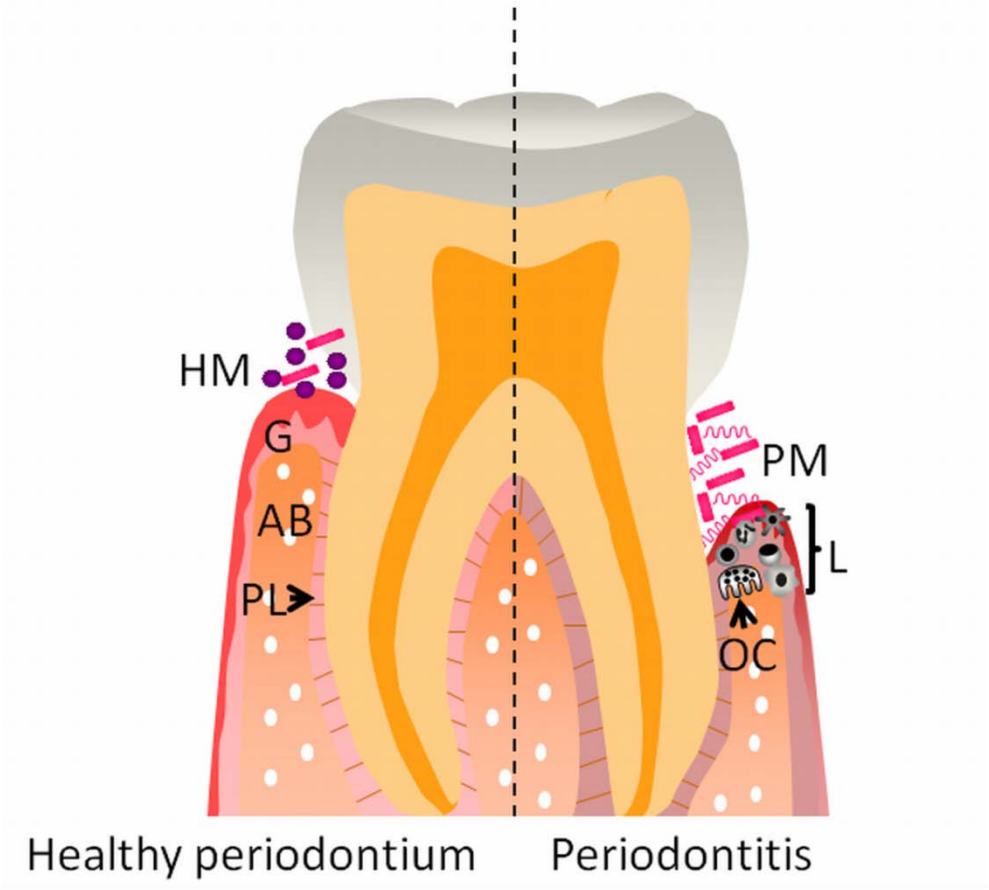

Figure 1. Healthy periodontium versus periodontitis

HM: bacterial microbiota in health; PM: Periodontopathogens; G: Gingiva; AB: Alveolar bone; PL: Periodontal ligament; L: Infiltrating leukocytes; OC: Osteoclast.

2.2 Clinical model of disease progression

Chronic periodontitis has been proposed to progress in both a slow continuous manner and in short bursts of disease activity, followed by periods of disease inactivity or quiescence (Goodson et al., 1982; Goodson et al., 1984; Loe et al., 1978; Socransky et al., 1984).

Accordingly, periodontal tissue support is lost during short, acute episodes of disease activity or "bursts", followed by variable periods of quiescence. Thus, the longitudinal sequential examination of clinical attachment by periodontal probing is the most common clinical method to diagnose progressive periodontal disease (Reddy et al., 1997).

The tolerance method has been the most prevalent clinical approach to determine site-specific attachment level changes (Haffajee et al., 1983) because of its potential for early detection. The method is based on longitudinal clinical examinations and recording the attachment loss. Progression is defined as attachment loss ≥ 2 mm during a 2-month period at the site level (active sites) and at least 2 active sites must be identified to consider the patient as undergoing disease progression.

2.3 Etiopathogenesis of chronic periodontitis

2.31 Dental plaque

Bacterial biofilms represent the primary aetiological factor in the initiation of gingival inflammation and subsequent destruction of periodontal supporting tissues (Haffajee and Socransky, 1994). There is strong evidence that suggests that *Aggregatibacter (A.) actinomycetemcomitans, Porphyromonas (P.) gingivalis* and *Tannerella (T.) forsythia* are periodontal pathogens when they are present in sufficient numbers and in susceptible hosts (Consensus report. Periodontal diseases: pathogenesis and microbial factors, 1996).

P. gingivalis is a gram-negative, black-pigmented, strictly anaerobic asaccharolytic bacteria that has previously been involved in periodontal disease progression (Holt et al., 1999). *P. gingivalis* produces an array of potential virulence factors (Yilmaz, 2008), such as

lipopolysaccharide (LPS), capsule polysaccharides, hemagglutinins, fimbriae and cysteine proteases known as “gingipains” (Hajishengallis, 2009). Despite the reported differences in the prevalence of periodontal pathogens among ethnically or geographically distinct populations, *P. gingivalis* has shown to be the most frequent periodontal pathogen associated with periodontitis in the Chilean population (Herrera et al., 2008; Lopez et al., 2004). Additionally, significantly elevated levels and mean percentages of *P. gingivalis* have been reported in active periodontal lesions compared with inactive ones (Lopez, 2000; Silva et al., 2008) and have been suggested to predict disease progression (Byrne et al., 2009).

2.32 Host response: General considerations

Although chronic bacterial exposure is a prerequisite for the occurrence of periodontal tissue destruction, its presence alone is not sufficient to explain disease initiation and progression (Grossi et al., 1994). In fact, the major component of soft- and hard- tissue destruction associated with periodontitis results from the activation of the host’s immune-inflammatory response to the bacterial challenge (Gemmell et al., 2002). On the basis of histopathological features, infection induces vascular and cellular changes with the formation of an inflammatory infiltrate, destruction of alveolar bone and apical migration of junctional epithelium (Kinane, 2001). The inflammatory process developed in periodontitis may be divided into three phases: acute, immune response and the chronic phase (Offenbacher, 1996). The transition from gingival health to early inflammation is characterised by a local increase in vascular permeability, redness, swelling and the recruitment and activation of polymorphonuclear neutrophils (PMNs) (Delima and Van

Dyke, 2003). Posterior influx of macrophages and antigen-presenting cells present the foreign antigens to T lymphocytes during the subsequent immune response phase, followed by the expansion of antibody-secreting plasma cells resulting in the development of a chronic lesion (Gemmell and Seymour, 2004). As a result of immune activation, the active expression of osteolytic cytokines, such as interleukin (IL)-1β and tumour necrosis factor alpha (TNF)-α, stimulates the alveolar bone resorption and collagen destruction via tissue-derived MMPs, a major pathway for the breakdown of bony and soft connective tissue associated with periodontal activity (Buduneli and Kinane, 2011; Offenbacher, 1996).

2.4 Gingival crevicular fluid

In physiologic conditions, gingival crevicular fluid (GCF) is a transudate composed of molecules derived from serum. Host cells from gingival tissue, inflammatory leukocytes and bacteria from subgingival plaque can be harvested non-invasively from the gingival sulcus. With the onset of inflammation, increased vascular and epithelial permeability permits the extravasation of high molecular proteins from general circulation and periodontal tissues; thus, GCF shifts to an inflammatory exudate (Adonogianaki et al., 1996; Buduneli and Kinane, 2011; Curtis et al., 1990; Oringer et al., 2002). In healthy subjects and under resting conditions, GCF flow rates correspond to approximately 0.1 μL/min per tooth, but these rates can increase up to fivefold in subjects affected by gingivitis and periodontitis (Curtis et al., 1990; Pisano et al., 2005; Uitto et al., 2003). During the disease, GCF contains molecules involved in the destructive process, and these

molecules show significant potential as a source of disease biomarkers that might reflect soft and hard tissue catabolism (Loos and Tjoa, 2005).

2.5 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are genetically distinct but structurally related zinc-dependent metalloendopeptidases. MMPs degrade extracellular matrix and further potentiate proteolysis and inflammation by processing bioactive non-matrix substrates, such as cytokines, chemokines and growth factors, and by activating other MMPs (Folgueras et al., 2004; McQuibban et al., 2001; McQuibban et al., 2002; Overall et al., 2002; Sorsa et al., 2004; Sorsa et al., 2006). MMPs share a similar basic structure composed of three domains, the pro-peptide, catalytic and the hemopexin-like domain (Kessenbrock et al., 2010). The 23 MMPs expressed in humans can be classified into different subgroups based on their primary structures and substrate specificities: collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), membrane-type MMPs (MT-MMPs, MMP-14, -15, -16, -17, -24 and -25) and other MMPs (Folgueras et al., 2004), (Figure 2.).

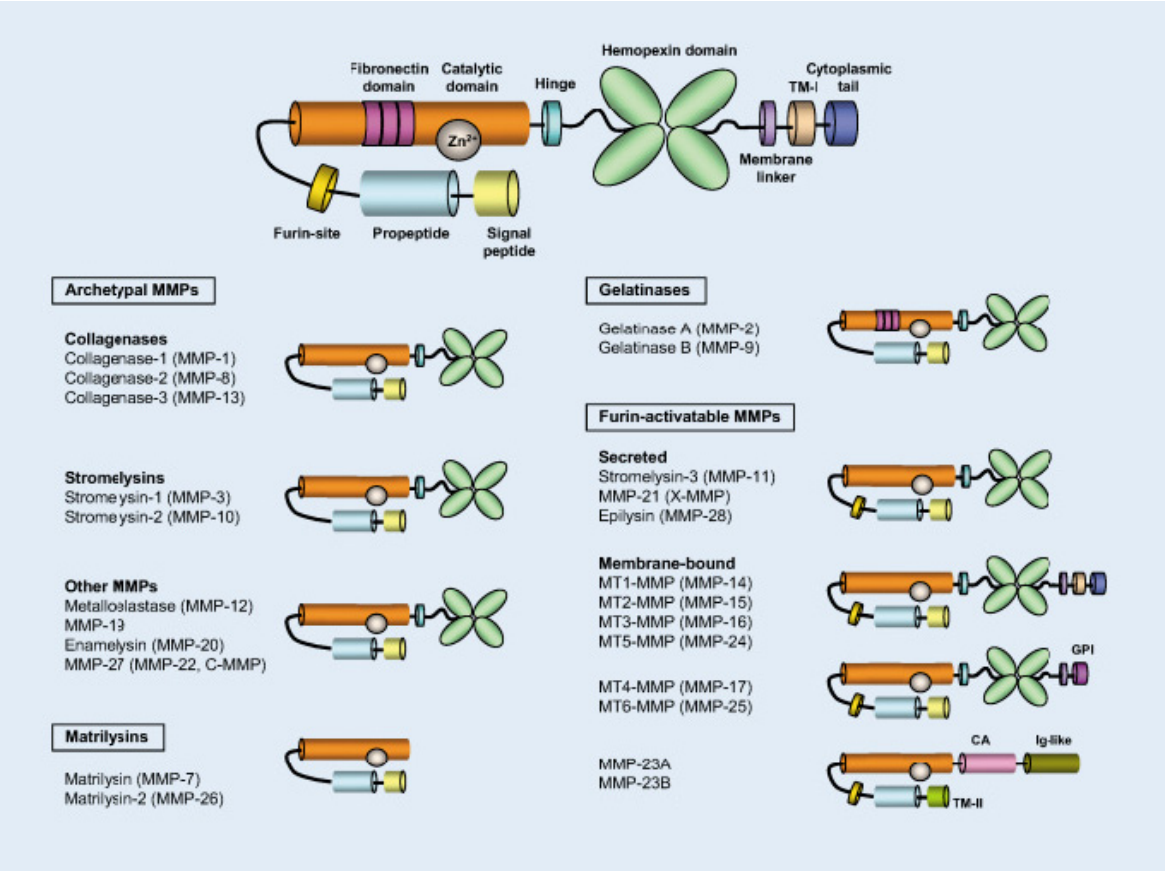

Figure 2. Structural classification of MMPs

Reproduced with permission from The International Journal of Developmental Biology 2004;48:411-24 (Folgueras et al., 2004).

2.51 Regulation of MMP activity

MMP proteolytic activity is subjected to a complex regulation through gene expression, compartmentalisation, and zymogen conversion, as well as the action of specific inhibitors. MMPs are initially expressed in an enzymatically inactive state because of the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site. The disruption of this interaction (cysteine switch) by proteolytic removal or chemical modification results

in enzyme activation. There are several proteinase cascades that mediate and amplify MMP activation, such as plasmin, furin and active MMPs, as well as chemical activators, such as reactive oxygen species (ROS) (Kessenbrock et al., 2010; Sorsa et al., 1992; Weiss et al., 1985). Once activated, the most important physiological inhibitors are tissue inhibitors of MMPs (TIMPs) -1, -2, -3 and -4 (Kessenbrock et al., 2010), Thus, the pathophysiological significance of increased MMP expression in periodontitis will rely on their regulation by activating enzymes or compounds and endogenous inhibitors.

2.52 Collagenolytic MMP involvement in chronic periodontitis

Pathologically enhanced expression and activation of host MMPs over the protective shield provided by their TIMPs induced by bacterial infection and cytokines is a well characterised feature in chronic periodontitis. Many inflammatory mediators have been proposed as potential biomarkers, including MMPs; however, most of them demonstrate limited usefulness because they reflect periodontal inflammation rather than disease progression (Loos and Tjoa, 2005; Sorsa et al., 2006). Because type I collagen is the primary extracellular matrix component of soft and hard periodontal tissues, collagen degradation is one of the key factors in uncontrolled destructive lesions, driving the attention towards collagenolytic MMPs (Golub et al., 1997). The determination of MMP levels and activities in disease progression may aid in the identification of susceptible sites and individuals under the risk of developing periodontal attachment loss.

Five decades ago, the first collagenolytic enzyme was discovered (Gross and Lapiere, 1962), and it was promptly identified in human gingiva (Fullmer and Gibson, 1966; Gibson

and Fullmer, 1966) and GCF (Golub et al., 1976) in association with the degree and severity of gingival inflammation (Overall and Sodek, 1987). Since then, growing evidence has supported that collagenases along with other cooperative MMPs play a central role in periodontal tissue destruction (Sorsa et al., 2004). The major collagenolytic MMPs associated with the severity of periodontal inflammation and disease are collagenase-2 (MMP-8) and collagenase-3 (MMP-13), whereas collagenase-1 (MMP-1) is more related to periodontal tissue turnover under physiological conditions (Kiili et al., 2002; Makela et al., 1994; Sorsa et al., 1988; Sorsa et al., 2004; Sorsa et al., 2006; Tervahartiala et al., 2000; Uitto et al., 2003). GCF MMP-8 accounts for the bulk of collagenases, followed by MMP-13 (Golub et al., 1997; Golub et al., 2008). During periodontal inflammation, latent proMMPs might become active by the cooperative action of other MMPs, such as MMP-14, ROS and microbial proteases (Holopainen et al., 2003; Ilgenli et al., 2006; Knauper et al., 1996; Leeman et al., 2002).

A reduction in GCF total collagenase activity has been associated with the improvement of clinical parameters and the decrease of bone resorption fragment carboxyterminal telopeptide of type I collagen (ICTP) in subantimicrobial dose doxycycline (SDD)-treated post-menopausal women exhibiting periodontitis and systemic osteopenia during a two-year follow-up period (Golub et al., 2008). This finding supports a role for collagenases in bone loss and thus in disease progression, which can be reflected in GCF.

Despite the genetic background underlying periodontal diseases, MMP-1, -2, -9 and -13 gene polymorphisms studies undergone in different ethnic populations have been conducted without finding any associations with the susceptibility to chronic periodontitis, except for

the MMP-1 polymorphic allele forms (Chen et al., 2007; Pirhan et al., 2008; Pirhan et al., 2009; Ustun et al., 2008).

2.53 MMP-8 in periodontal destructive lesions

Matrix metalloproteinase-8 (MMP-8) is the major collagenolytic MMP in GCF, and elevated levels have been widely associated with the severity of periodontal inflammation and disease (Emingil et al., 2004; Kiili et al., 2002; Kinane et al., 2003; Mantyla et al., 2003; Sorsa et al., 2006; Sorsa et al., 2010). MMP-8 not only degrades the primary extracellular matrix components of the periodontium, but also regulates the immune response; MMPs influence the bioavailability and biological activity of cytokines by proteolytic processing of non-matrix bioactive molecules, thus modifying their biological activity or levels (Gutierrez-Fernandez et al., 2007; Nilsson et al., 2009).

The elevation of active MMP-8 has been previously associated with the conversion of gingivitis to periodontitis and the progression of established periodontitis (Lee et al., 1995; Mantyla et al., 2006; Romanelli et al., 1999). Repeatedly elevated GCF MMP-8 concentrations determined by longitudinal monitoring of periodontal responses during the maintenance phase after scaling and root planning (SRP) were reported to be associated with a lack of improvement in clinical parameters (Mantyla et al., 2006). Additionally, increases in MMP-8 and IL-1β during the first year of periodontal maintenance have been reported to be associated with increased odds of subsequent attachment loss; whereas elevated levels of pyridinoline cross-linked ICTP at baseline resulted in increased odds of alveolar bone density and height loss driven primarily by a placebo group related to SDD

adjunctive therapy experimental group. No associations were found between MMP-8 levels and bone loss (Reinhardt et al., 2010). Overall, MMP-8 levels could reflect soft periodontal tissue destruction and periodontal response to treatment (Sorsa et al., 2010).

In spite of the well-known association between high MMP-8 levels and periodontitis, its biological functions are not completely clear (Gutierrez-Fernandez et al., 2007). MMP-8 deficient mouse models reveal an impaired inflammatory response, characterised by abnormal neutrophil infiltration and expression of cytokines, such as transforming growth factor (TGF)-β (Gutierrez-Fernandez et al., 2007; Korpi et al., 2009). The recent development of the *P. gingivalis*-induced periodontitis MMP-8-deficient mice model unexpectedly showed extensive alveolar bone resorption (Kuula et al., 2009).

Additionally, the destructive periodontal disease has been associated with and increased risk of cardiovascular complications. Recently, MMP-8 has been implicated in atherosclerotic plaque destabilisation by the thinning of the protective fibrous cap (Herman et al., 2001). *P. gingivalis*-induced experimental periodontitis in MMP-8 null mice have altered lipoprotein profiles, compared with wild types (Kuula et al., 2009). Similarly, *A. Actinomycetemcomitans*-infected mice displayed proatherogenic lipoprotein profiles, increased C-reactive protein (CRP), MMP-9 and lipid metabolism in serum of Apolipoprotein E-deficient mice, known to promote the development of atherosclerosis (Tuomainen et al., 2008).

2.54 MMP-13 in periodontal destructive lesions

MMP-13 was first cloned from breast carcinoma (Freije et al., 1994) and is considered to play an important role in skeletal biology (Stahle-Backdahl et al., 1997). MMP-13 is expressed by sulcular epithelial cells, endothelial cells, macrophage-like cells, fibroblasts, plasma cells and osteoblasts (Hernandez et al., 2006; Nakamura et al., 2004; Rydziel et al., 2000). Longitudinal studies in progression of chronic periodontitis, which was addressed clinically by the tolerance method, demonstrated that active sites had significantly elevated MMP-13 activity (Hernandez et al., 2006). On the basis of these data, MMP-13 might be associated with the progression of periodontal attachment loss.

Evidence suggests that MMP-13 might initiate bone resorption by activating osteoclasts (Holliday et al., 1997) and proMMP-9 *in vitro* (Knauper et al., 1997b). Active MMP-9 further digests denatured collagen derived from MMP-13 activity (Hill et al., 1995) and is thought to act over preosteoclast recruitment as well as to activate proMMP-13 and proMMP-2. Additionally, proMMP-13 can also be activated *in vitro* by active MMP-14, MMP-13 and MMP-2 (Knauper et al., 1996). Soluble forms of all of these MMPs have been described in periodontitis GCF and gingival tissue (Ilgenli et al., 2006; Tervahartiala et al., 2000), where they might act alone or by assembling proteolytic cascades.

2.6 Myeloperoxidase

Neutrophil-derived myeloperoxidase (MPO) is contained in primary (azurophilic) granules from neutrophils (Kowolik and Grant, 1983) and catalyses the formation of hypochlorous

acid (HOCl), a powerful antibacterial agent, which reflects the strength of oxidative stress (Wei et al., 2004).

MPO GCF levels have previously been associated with the severity of periodontitis (Wei et al., 2004). Similarly, MMP-8 and MMP-9 are primarily released from neutrophils in a latent form and can be induced and activated during periodontal inflammation by host inflammatory mediators, such as TNF-α, IL-1β, ROS and MPO-produced hypochlorous acid (Saari et al., 1990), as well as microbial and host-derived proteases (Sorsa et al., 1992). MPO can inactivate pathogenic microbes by generating reactive oxygen species, oxidatively activate latent proMMP-8 and -9, as well as inactivate TIMPs (Saari et al., 1990; Spallarossa et al., 2008; Wang et al., 2007). Thus, MPO can also oxidatively potentiate MMP-cascades in periodontal tissue destruction, becoming potentially deleterious (Saari et al., 1990).

2.7 Cytokines

Cytokines are soluble proteins that bind to specific receptors on target cells; they have transient expression and pleiotropic effects on target cell types. They play a fundamental role in inflammatory diseases, such as periodontitis (Preshaw and Taylor, 2011). The progression of periodontitis and its clinical outcome depend on the host's immune response, leading to the recruitment of inflammatory cells, the generation of cytokines, the activation of osteoclasts (Seymour et al., 1993) and collagen destruction via tissue-derived matrix metalloproteinases (Offenbacher, 1996).

Cytokines regulate MMP expression. MMPs influence in turn the immune response regulating the biological activity of cytokines via proteolytic processing of non-matrix bioactive molecules (Sorsa et al., 2004; Tester et al., 2007; Van Lint et al., 2005). Furthermore, recent reports have demonstrated changes in cytokine levels associated with MMP expression and activity (Gutierrez-Fernandez et al., 2007; Nilsson et al., 2009).

Chemokines are a superfamily of structurally related chemoattractant cytokines that direct the migration of leukocytes, regulate inflammation and cell trafficking. Among them, two primary families are classified based on the spacing of the first two cysteine residues: (1) the CXC chemokine family, such as IL-8/CXCL8, mouse lipopolysaccharide (LPS)-induced CXC chemokine (LIX/CXCL5) and stromal-derived factor (SDF)-1/CXCL12; and (2) the CC chemokine family, which include the monocyte chemoattractant proteins (MCP)-1 to -4. In general terms, CXC chemokines attract PMN during the initial phases of inflammation, whereas CC chemokines target multiple leukocyte subsets (McQuibban et al., 2001; McQuibban et al., 2002).

2.71 Mouse lipopolysaccharide (LPS)-induced CXC chemokines

PMN are strategically localised between the bacterial biofilm and the junctional epithelium, where they represent the first line of defence among all leukocytes (Moughal et al., 1992; Tonetti et al., 1998). It is generally accepted that PMN contribute to a protective response against subgingival plaque, maintaining the integrity of attachment apparatus, whereas alterations in neutrophil function are associated with severe periodontal disease phenotypes (Kebschull et al., 2009). Mouse lipopolysaccharide (LPS)-induced CXC chemokine

(LIX/CXCL5) is the most abundant and potent murine chemoattractant for neutrophils *in vitro* and *in vivo*, representing the sole homologue of two closely related human chemokines, CXCL5 and CXCL6 (Kebschull et al., 2009; Kornman et al., 1997; Ruddy et al., 2004).

MMP-8 deficient mice have shown decreased levels of PMN infiltration resulting from reduced LIX/CXCL5 mobilisation from the extracellular matrix (Van Lint et al., 2005). Recently, the differential expression of LIX/CXCL5 human homologue granulocyte chemotactic protein-2 (GCP-2/CXCL6) was reported to be upregulated in periodontal disease, which correlated positively with clinical parameters and periodontal pathogens (Kebschull et al., 2009).

2.72 Stromal-derived factor-1

Stromal-derived factor (SDF)-1/CXCL12 belongs to the CXC family of chemokines. Activation of its unique receptor CXCR4 is essential for its many functions, such as hematopoietic cell homing to bone marrow, promotion of chemotaxis, early development, cell function, bone resorption, aid in the survival of human osteoclasts and their monocytic precursors (in a manner similar to the early effects of the M-CSF+RANKL system), and enhance PMN migration. Recently, increased GCF SDF-1/CXCL12 levels have been associated with chronic periodontitis and periodontal tissue response to conventional treatment (Havens et al., 2008).

2.73 Receptor activator of NF-κB ligand

The receptor activator of the NF-κB ligand (RANKL) is a crucial mediator required, along with the permissive colony stimulating factor (CSF)-1, for the full development of osteoclast precursors into mature multi-nucleated bone-resorbing cells (Fuller et al., 2007; Wright et al., 2005). RANKL exerts its biological effects directly through binding to its receptor RANK, inducing osteoclast differentiation, maturation, activation, and inhibiting their apoptosis (Lacey et al., 1998; Theill et al., 2002); whereas osteoprotegerin (OPG) is a decoy receptor that inhibits osteoclastogenesis through binding of RANKL (Simonet et al., 1997). Inflammation and bone resorption during periodontitis have been widely associated with high RANKL levels and an augmented RANKL/OPG ratio (Vernal et al., 2006; Wright et al., 2005; Yu et al., 2003). Moreover, RANKL levels were found to be significantly higher in GCF from active sites versus inactive sites during progressive periodontitis (Vernal et al., 2004), suggesting its involvement in destructive periodontitis.

3. HYPOTHESIS AND AIMS OF THE STUDY

Many potential biomarkers have been proposed in chronic periodontitis, but none of them, either alone or in combination, can be used to prevent irreversible periodontal breakdown. The characterisation of collagenolytic MMP levels and interactions in progressive periodontal lesions may aid in the development of chair-side point of care diagnostics, treatment decisions and future drug development. We propose that enhanced collagenase-mediated proteolytic networks, involving enzyme activators, inhibitors and non-matrix bioactive substrates, are associated with chronic periodontitis progression in diseased periodontal tissues and are reflected in gingival crevicular fluid (GCF). Therefore, they might be utilised as potential biomarkers for disease progression.

3.1 Aims of the study:

1. To characterise the levels and relationships between MMP-13 to its major endogenous inhibitor TIMP-1 in GCF and gingival tissue from clinically progressive chronic periodontitis subjects and controls.

2. To associate MMP-13 activity with levels of ICTP, TIMP-1 and MMP-9 activation rates in progressive chronic periodontitis.

3. To identify potential oxidative (MPO) and proteolytic (MMP-14) interactions of MMP-8 in GCF from clinically progressive chronic periodontitis at baseline and after conventional treatment.

4. To characterise the levels and tissue localisation patterns of potential cytokine MMP-8 substrates in *P. gingivalis*-induced experimental periodontitis in MMP-$8^{-/-}$ mice v/s wild type (WT) mice.

4. MATERIALS AND METHODS

4.1 Human studies (I-III)

4.11 Study subjects (I-III)

Patients with moderate to severe chronic periodontitis were enrolled in the study and were followed until they developed progression of the clinical disease. Patients were selected from the Center of Diagnostics and Treatment of Northern Metropolitan Health Services, Santiago. The inclusion criteria were a minimum of 14 natural teeth (excluding 3rd molars), at least 10 posterior teeth, at least 5-6 teeth had sites with probing depth ≥ 5mm, with attachment loss ≥ 3 mm and detectable bone loss via radiography; in addition, they must not have received previous periodontal treatment at the time of examination. Periodontally healthy volunteers also entered the study for control GCF collections. Exclusion criteria were as follows: history of systemic disorders, such as diabetes mellitus, osteoporosis and medications known to influence periodontal tissues, pregnancy or lactating females, and patient who had received antibiotic, anti-inflammatory, anticoagulant or hormonal drugs within the past six months prior to the study.

4.12 Clinical examinations

Probing depth (PD), clinical attachment loss (CAL), and dichotomous measurements of supragingival plaque accumulation (PA) and bleeding on probing from the base of the crevice (BOP) were recorded at six sites around the tooth.

Disease activity was defined clinically by the tolerance method (Haffajee et al., 1983). At the site level, active sites exhibited attachment loss ≥ 2.0 mm during the 2-month period.

Inactive sites were defined as those sites with probing depth and BOP equivalent to active sites, but without attachment loss during the same period. The patients were considered to undergo disease progression if they had a minimum of 2 active sites. Clinical parameter measurements were monitored at baseline, 2 and 4 months.

When a patient was diagnosed as undergoing disease progression, GCF or gingival samples (when periodontal surgery was indicated) from both active and inactive sites were immediately taken at baseline and 2 months after treatment. Periodontal therapy consisted of scaling, root planning, and instruction of oral hygiene. The Institutional Reviews Board-approved informed consent forms were signed by all of the study subjects. Upon the detection of disease progression, the patients immediately entered the treatment phase.

4.13 Gingival crevicular fluid samples (I, II, III)

After isolating the tooth with a cotton roll, supragingival plaque was removed, and GCF was collected for 30 s with paper strips (Proflow, Amityville, NY, USA). The volume of GCF was measured using a calibrated Periotron 8000 (Proflow, Amityville, NY, USA). GCF was extracted twice by centrifugation at 12.000 rpm for 5 min at 4°C in a standard volume of elution buffer containing 50 mM Tris-HCl pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.01% Triton X-100 and kept at -80°C, until MMP enzyme forms, activity and mediator levels were analyzed.

4.14 Gingival tissue samples (I)

An incision was made with a surgical blade through the gingival crevice to the alveolar crest. After washing, samples were homogenised in 0.15 M NaCl with a proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany), centrifuged at 13,000 x g for 6 min at 4°C and kept under -80° until analysed for total levels of MMP-13 and TIMP-1.

4.15 Gingival explant cultures (II)

Another group of gingival biopsies were prepared for explant cultures in 24-well plate with supplemented DMEM in a tissue/media ratio of 100:1 (w/v). After testing different MMP-13 concentrations for 0.5-24 incubation hours, explant cultures were treated with or without recombinant MMP-13 (Chemicon, Temecula, California, USA) at a ratio of 1/3.000 (w/v) for 1 h and 24 h and inactivated with 15 mM EDTA. Additional controls were made by adding 10 µM CL-82198 (EMD Biosciences, San Diego, CA), a selective synthetic MMP-13 inhibitor, following the manufacturer's recommendations. Culture supernatants were reserved for gelatine zymography assay.

4.16 Determination of total protein concentration (I)

Tissue homogenates and GCF total protein concentrations were measured by a Micro BCA kit (Pierce, Rockford, USA), following the manufacturer's recommendations. Results were expressed as mg/mL of total protein content.

4.17 Immunoblotting of MMP-8, MMP-13, MMP-14, MPO and TIMP-1 (I, III)

MMP-8, MMP-14, MPO and TIMP-1 immunoreactivities in GCF and/or tissue homogenates were determined by immuno-Western blotting in non-reducing conditions using a specific rabbit polyclonal anti-human MMP-8 antibody (1:500 dilution as previously described), (Hanemaaijer et al., 1997; Sorsa et al., 1994; Sorsa et al., 1999; Tervahartiala et al., 2000), rabbit polyclonal anti-human MMP-14 antibody (1:500 dilution), (Biogenesis, Poole, England) and monoclonal anti-human MPO antibody (1:1000 dilution), (R&D Systems, Inc. Minneapolis, MN, USA). A positive control for MPO, consisting of recombinant protein, was added (R&D Systems, Inc. Minneapolis, MN, USA). Immuno-Western blots were performed under reducing conditions using monoclonal anti-human

MMP-13 and TIMP-1 antibodies (Chemicon, Temecula, California, USA), which were both diluted 1:200.

After confirming the mono specificity of MMP-13 and TIMP-1 antibodies by immuno-Western blots, aliquots of GCF and tissue homogenates were applied in duplicates onto the PVDF membranes, while the following steps were performed as described for immuno-Western blots. The quantification was conducted with a Bio-Rad model GS-700 Imaging Densitometer using the Quantity-One–program (Bio-Rad, Hercules, CA, USA). The results were expressed as arbitrary units (au/mL) and per mg of total protein.

4.18 Determination of MMP-8, MPO, TIMP-1 and ICTP protein levels (II, III)

MMP-8 protein levels were measured by a time-resolved immunofluorometric assay (Hanemaaijer et al., 1997). MPO was determined by enzyme-immunosorbent assay (Immundiagnostik AG, Bensheim, Germany) and TIMP-1 and MMP-8 additionally, by the Biotrak ELISA system (GE Healthcare, Amersham, Slough, Berkshire, UK). An enzyme immunoassay (EIA) was employed for measurement of ICTP levels (Orion Diagnostica, Espoo, Finland); each technique was performed by following the manufacturer's recommendations. Protein concentrations were obtained from a standard curve and were expressed as ng/mL of eluted GCF.

4.19 Activity assays:

MMP-13 activity assay (II)

The MMP-13 activity was measured in aliquots of GCF samples by the "Fluorokine E" activity fluorescent assay (R&D Systems, Inc. Minneapolis, USA), according to the manufacturer's recommendations. GCF aliquots and standards were added to the wells, and

any MMP-13 present was bound to the immobilised MMP-13 antibody. After washing, a fluorogenic substrate linked to a quencher molecule was added, allowing a fluorescent signal after cleavage by active MMP-13. The enzyme activity was expressed as ng of the fluorescent product (ng FP) per mL of eluate.

MMP-14 activity assay (III)

The MMP-14 active enzyme was measured in GCF aliquots using an MMP-14 Biotrak activity assay system (GE Healthcare, Amersham, Slough, Berkshire, UK), following the manufacturer's recommendations. Briefly, the standards (MMP-14) and the samples were added to a microplate, and MMP-14 was bound to the immobilised MMP-14 antibodies in the wells. The assay used the proform of a detection enzyme with an artificial sequence that was activated by captured active MMP-14 and was measured by adding a specific chromogenic peptide substrate and read via a microplate spectrophotometer at 405 nm. The total (active+proenzyme) MMP-14 in the samples was also measured by adding aminophenylmercuric acetate (APMA), (Sigma, St. Louis, MO, USA). Results were expressed as ng/mL of eluate.

Gelatin zymography (II)

To determine the MMP-9 and -2 activities, aliquots of supernatants from explant cultures were run under non-reducing denaturing conditions on 10% polyacrylamide gels containing 1 mg/mL gelatine (Merck, Darmstadt, Germany); the gels were washed, incubated in developing buffer (20 mM Tris pH 7.4 and 5 mM $CaCl_2$) for 17 h, stained and destained. The densitometric analysis was performed as described above and results were expressed as arbitrary units of density/mL of supernatant.

4.2 Animal studies (IV)

4.21 Animals

Study animals and specimens were previously manipulated and prepared by Kuula et al. (2009) and included 14-week-old male mice maintained in the experimental animal facilities of the University of Oulu, Oulu, Finland. The MMP-8$^{-/-}$ knock out (KO) mice from a mixed C57BL/6J/129 background (Balbin et al., 2003) and wild-type (WT) mice littermates, resulting from intercrossing of heterozygous mice, were included as controls (Balbin et al., 2003; Korpi et al., 2008; Korpi et al., 2009). Experiments were conducted in accordance with the guidelines of the Animal Experimentation Committee of University of Oulu, Oulu Finland.

4.22 Induction of experimental periodontitis

The experimental groups were assigned as follows: WT-infected (n = 10), WT uninfected (n = 8), MMP-8 KO infected (n = 12) and MMP-8 KO uninfected (n = 10) (total N = 40). Statistical power analysis, based on a pilot study, was previously performed to determine an appropriate sample size.

To eliminate the native flora from the oral cavity, the mice received 20 mg of kanamycin and 20 mg of ampicillin in 1mL of sterile water twice daily for 3 days and were then allowed to clear it from their systems for 4 days prior to oral inoculation with *P. gingivalis (*strain ATCC33277, American Type Culture Collection). A suspension of approximately 0.1-0.2 mL of carboxymethyl cellulose with viable *P. gingivalis* was swabbed intraorally twice a day for

3 days, while negative controls received saline. Mice were killed 30 days after the last inoculation by cervical dislocation. The skulls were processed for routine HE and immunohistochemical analyses.

4.23 Immunohistochemistry

Immunohistochemistry was used to analyse the cytokine presence and levels with rabbit anti-LIX/CXCL5 (Preprotech, EC, London, U.K.), mouse anti-SDF-1/CXCL12 (R&D Systems, Inc. Minneapolis, USA) and goat anti-RANKL (R&D Systems, Inc. Minneapolis, USA) antibodies; the presence of the studied cytokines was visualised with a Vectastain Elite ABC kit (Vector Labs, Burlingame, CA, USA) using biotinylated secondary antibodies and 3-amino-9-ethylcarbazole (AEC) as chromogen. Randomly selected images were acquired using an Olympus BX61 microscope with an Olympus DP50 camera, as well as UPlanFl 40x/0.75 NA and 20x/0.50 NA objectives. Immunoreactive mesenchymal cells from total cells and the immunoreactive area of gingival epithelia from total gingival epithelia, respectively, were analysed with a macro written for the public domain ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009).

4.3 Statistical analysis

Differences regarding dichotomic measurements were analysed by a chi-squared test; comparisons between two related groups, using a paired t-test or Wilcoxon test depended on the data distribution, whereas comparisons between two unrelated groups were analysed with

an unpaired t-test and Mann-Whitney test. An ANOVA with Bonferroni´s or Tukey`s post hoc test was performed for >2 independent groups and for comparisons with parametric data distribution. Pearson`s or Spearman's correlation was applied to determine the association between variables. Comparisons between active, inactive –from progressive subjects– and healthy subjects were analysed separately using the linear mixed model. In each model (MMP, ICTP, TIMP), comparisons between groups were performed with an F-test, and denominator degrees of freedom were computed by the Kenward method, and further correction for the multiple testing was applied using the Tukey adjustment.

A p value <0.05 was considered statistically significant. Statistical analyses were performed with Stata V10 software (StataCorp, Collage Station, TX, USA) and SAS proc Mixed (SAS Version 9.1, Copyright 1999-2001 SAS Institute Inc., Cary, NC, USA).

5. RESULTS

5.1 Human studies

5.11 Clinical parameters (I, II, III)

Clinical features from periodontitis patients versus controls differed only regarding to periodontal clinical parameters. In periodontitis patients, all clinical parameters improved significantly after conventional periodontal treatment.

5.12 GCF volumes and total protein concentrations in healthy and progressive periodontitis subjects (I)

Mean GCF volumes and total protein concentration significantly increased in progressive periodontitis patients (n = 21 actives and 21 inactives), when compared with healthy subjects (n = 11). The mean GCF volumes were similar among active and inactive sites and the mean total protein concentrations were significantly different among all groups, decreasing from active sites to healthy ones (Table 1.).

5.13 MMP-13 and TIMP-1 molecular forms in healthy and progressive periodontitis subjects (I)

MMP-13 detection was limited to disease GCF as immunoreactive bands of ~60 kDa (proform), 56 kDa (intermediate forms), 48 kDa (active enzyme) and fragments (25-35 kDa); TIMP-1 was identified as a 34 kDa immunoreactive protein.

5.14 MMP-13, ICTP and TIMP-1 levels in healthy and progressive periodontitis subjects (I, II)

MMP-13 protein levels were undetected in healthy GCF by either immunodot blot or immuno-Western blot, whereas all periodontitis sites showed positive results. A comparison of MMP-13 and TIMP-1 levels in tissue samples and GCF from active and inactive sites from progressive periodontitis subjects by immunodot blotting showed no significant differences among sites. Nevertheless, a trend to a positive correlation between TIMP-1 and MMP-13 was found in inactive sites ($r = 0.61$, $p = 0.2$), compared with an inverse correlation for active sites ($r = -0.5$, $p = 0.35$).

Regarding MMP-13 activity, even if there was a trend to increase from healthy to active sites in progressive patients, significant differences were found only for active sites that displayed the highest MMP-13 activity, compared with inactive and healthy sites. Similar changes were found for ICTP levels that showed the highest levels ($p<0.05$) in active sites versus inactive ones and healthy controls. TIMP-1 was not detected in all samples and percentages of detection, as well as mean levels, increased in healthy GCF compared with active and inactive sites, but the difference was significant only for percentages of detection (Table 1.).

5.15 MMP-13 and gelatinase activation in periodontitis-affected gingival tissue (II)

Adding of exogenous recombinant MMP-13 to gingival explants from periodontitis patients resulted in significantly increased proMMP-9 activation at 1 h and 24 h after enzyme addition and a trend towards higher MMP-2 activation rates. Almost no detectable active MMP-9 was

seen, despite the addition of MMP-13 when the CL-82198 MMP-13 selective inhibitor was used. Additionally, a strong positive correlation was found between the active MMP-9 and MMP-2 enzyme forms ($r = 0.84$, $p<0.0001$).

5.16 MMP-8, MMP-14 and MPO molecular forms in GCF from progressive periodontitis (III)

MMP-8 immunoreactivities were found as ~85 and ~64 kDa bands for pro and active PMN isoforms, respectively, ~55 and ~48 kDa for mesenchymal proform and active enzymes, respectively, for complexes of ≥100 kDa and fragments≤ 46 kDa. MMP-14 soluble forms were found as ~50 kDa bands, whereas complexes and fragments were also detected. MPO immunoreactivities were identified at ~75, 90, 130 and 160 kDa. All enzyme forms were detected in all screened samples from both active and inactive sites.

5.17 MMP-8, MMP-14 and MPO levels in GCF from progressive periodontitis at baseline and after treatment (III)

Densitometric analysis of MMP-8 immunoreactivities demonstrated that PMN-type MMP-8 forms predominated over the mesenchymal forms. Among all MMP-8 forms, active enzymes and the percentages of activation for both PMN and mesenchymal isotypes tended to increase at inactive sites, whereas all MMP-14 forms tended to increase at active sites, except for the complexes. Similarly, the analysis of protein levels for MMP-8, total and active MMP-14 and MPO demonstrated that only active and total MMP-14 levels tended to increase in active sites, but none of the differences were significant (Table 1.).

The total levels of MMP-8 and MPO were measured in progressive periodontitis sites at baseline and after periodontal treatment, showing significant reductions, except for MMP-8 in active sites.

5.18 Association between MPO levels and MMP-8 in GCF (III)

A positive correlation was found between total MPO with total MMP-8 ($r = 0.74$ inactives; $r = 0.92$ actives, $p<0.05$) and with its active forms (PMN isoform, $r = 0.77$; mesenchymal isoform, $r = 0.69$; $p<0.05$). This association was lost in inactive sites after periodontal treatment, but still remained for active sites.

5.2 Animal studies: *P. gingivalis*-induced experimental periodontitis in MMP-8 null mice and controls (IV)

Typical histopathological changes associated with chronic periodontitis were observed in *P. gingivalis*-infected mice. No differences were found in cytokine distribution by tooth localisation ($p>0.05$).

5.21 LIX/CXCL5 expression

LIX/CXCL5 was consistently expressed in the epithelia of gingival papilla from all studied mice; it was significantly reduced in the *P. gingivalis*-infected KO group compared with infected wild-type controls, with levels comparable to non-infected mice (Table 2.).

5.22 SDF-1/CXCL12 and RANKL expression

SDF-1/CXCL12 and RANKL expression patterns were more ubiquitous in periodontal tissues than LIX/CXCL5, but were almost restricted to *P. gingivalis*-infected groups. Moreover, the frequencies of detection for both cytokines were significantly higher in infected groups compared with uninfected ones (Table 2.). SDF-1/CXCL12 was primarily expressed in periodontal ligaments and the strongest staining was localised to the vascular channels close to areas of alveolar bone resorption. The most striking RANKL expression was localised to the periodontal ligament and osteoclasts surrounding the alveolar crest. SDF-1/CXCL12 and RANKL levels showed no significant differences between KO and WT mice, but a significant positive correlation was found between both cytokines in all study groups ($r = 0.40$, $p=0.01$).

Table 1. Summary table of examined potential biomarkers in GCF from progressive periodontitis patients at baseline and healthy subjects

	Active	Inactive	Controls	N	p
GCF mean volume (μL)	0.94±0.21	0.92±0.18	0.22±0.13	53	**<0.05**
GCF Mean protein concentration (mg/mL)	0.68±0.059	0.37±0.059	0.11±0.081	53	**<0.05**
MMP-13 activity (ng FP/mL)	1.49±0.46	1.17±0.20	1.03±0.18	53	**<0.05**
ICTP (ng/mL)	0.49±0.21	0.31±0.15	0.24±0.13	53	**<0.05**
TIMP-1 (% cases)	47.60%	76.20%	100%	53	**<0.05**
TIMP-1 (ng/mL)	0.319±0.09	0.286 ±0.10	0.673± 0.49	53	>0.05
MMP-8 (ng/mL)	119.35 (196.92)	153.86 (185.52)	---	50	>0.05
PMN MMP-8 active (au/mL)	0.52 (0.80)	0.39 (0.92)	---	28	>0.05
Mesenchymal MMP-8 active (au/mL)	0.45 (0.36)	0.15 (0.55)	---	28	>0.05
MPO (ng/mL)	287.94 (675.79)	660.05 (789.81)	---	50	>0.05
MMP-14 active enzyme (ng/mL)	6.89 (3.31)	5.57 (7.55)	---	50	>0.05
MMP-14 total (ng/mL)	18.80 (33.07)	16.82 (15.22)	---	50	>0.05

Values expressed as means ± SD or medians (IQR). GCF mean volume: Controls vs. inactives and actives $p<0.05$; inactives vs. actives $p>0.05$; GCF mean protein concentration: Actives vs. inactives and healthy $p<0.05$; Inactives vs. healthy $p<0.05$; MMP-13 activity: actives vs. inactives and controls $p<0.05$; inactives vs. healthy, $p>0.05$; ICTP levels: actives vs. inactives and controls $p<0.05$; inactives vs. healthy, $p>0.05$.

Table 2. LIX/CXCL5, SDF-1/CXCL12 and RANKL expression in MMP-8 knock out and wild-type mice infected with *P. gingivalis* and controls

	WT+Pg	KO+Pg	WT-Pg	KO-Pg	p
LIX/CXCL5	0.366±0.153*	0.222±0.175*	0.276±0.121	0.183±0.116*	**<0.005**
SDF-1/CXCL12 (%)	54.5	41.1	16.3	7.3	**<0.005**
RANKL (%)	80.0	65.0	12.8	6.4	**<0.005**

Results expressed as means ±SD of total positive area/total area per field or percentage (%) of immunostained sites from total examined sites. Asterisks represent the group pairs that displayed significant differences for the post-hoc test (WT+Pg vs. KO+Pg and KO-Pg, $p<0.05$).

6. DISCUSSION

Periodontitis is described as a multifactorial, irreversible and cumulative condition, initiated and propagated by bacteria and host factors (Buduneli and Kinane, 2011). Pathologically enhanced expression and activation of host MMPs induced by bacterial infection and cytokines are well-characterised features in chronic periodontitis. The resultant unhampered proteolysis might lead to periodontal damage and the perpetuation of the inflammatory response, favouring disease progression (Kessenbrock et al., 2010; Sorsa et al., 2006).

Chronic periodontitis progression is a continuous process that undergoes dynamic periods of activity and remission (Jeffcoat and Reddy, 1991; Kinane, 2001). Accordingly, periodontal support is lost during short, acute episodes of disease activity or bursts. Though the hallmark of disease activity is the loss of soft or hard tissue attachment to the tooth, longitudinal recording of clinical attachment by the tolerance method has been the most commonly used approach to diagnose progressive periodontal disease (Reddy et al., 1997). Consequently, analysis of GCF samples from active versus inactive progressive sites can provide a closer view of the proteolytic events involved in supporting tissue breakdown and surrogate biomarkers.

In this study, we show differences in protein composition among active, inactive and healthy sites, pointing towards increased proteolysis in the former sites. Enhanced collagenase levels and activity were found in chronic periodontitis and was associated with disease progression. Furthermore, enhanced collagenase-mediated proteolytic cascades seemed to interact with the immune-inflammatory components during the initiation and/or

progression of periodontitis. Based on these findings, GCF collagenase signatures can potentially be useful as biomarkers for disease progression.

GCF has widely been regarded as a key source of potential biomarkers in periodontitis. During periodontitis, GCF shifts from a transudate to an exudate; consequently, the GCF volume and composition change as result of protein extravasation from serum, local synthesis and release of catabolic products derived from periodontally diseased tissues (Adonogianaki et al., 1996; Curtis et al., 1990; Oringer et al., 2002). In the present study, the analysis of volume and total protein content in GCF demonstrated differences between healthy and chronic periodontitis subjects, as expected. During progressive periodontitis, we found that GCF from active sites showed similar volumes, but nearly as twice as much total protein concentration as inactive sites. In accordance with our results, increases up to fivefold in GCF volume have previously been reported in subjects affected by gingivitis and periodontitis, compared with healthy controls (Pisano et al., 2005). During chronic periodontitis, inflammatory changes in both active and inactive sites were reflected in increased GCF volume and total protein concentration when compared with healthy sites; Nonetheless, active sites were expected to undergo a current periodontal tissue breakdown compared with their inactive counterparts; this breakdown can be reflected in the increased protein content in GCF due to the release of protein mediators and periodontal matrix breakdown products, where collagens type I and III are the main constituents of both periodontal ligament and alveolar bone (Alpagot et al., 2001). GCF volume and protein content varied considerably according to the periodontal status. Because most of protein and volume variations in GCF from healthy and periodontitis sites can be attributed to the increasing of vascular permeability triggered by local inflammatory process, the current

results confirm that the more sensitive method of standardisation was through the expression of target proteins under fixed periods (30 s) of fluid collection (Golub et al., 1997; Lamster and Novak, 1992).

The initial cleavage of periodontal collagens by collagenolytic MMPs is believed to play a pivotal role over the loss of periodontal supporting tissues. Conversely, the collagenase inhibitor TIMP-1 has been related to connective tissue regeneration (Sorsa et al., 2006). Among collagenases, MMP-8 and MMP-13, along with the gelatinase MMP-9, are the major MMPs associated with the severity of periodontal disease (Leppilahti et al., 2010; Sorsa et al., 2004; Sorsa et al., 2006; Teles et al., 2008; Tervahartiala et al., 2000). In GCF from chronic periodontitis subjects, MMP-8 and MMP-9 are the predominant MMPs, whereas MMP-13 comprises only 3-4% of total collagenases (Kiili et al., 2002; Tervahartiala et al., 2000) and even less or no MMP-1 has been detected (Golub et al., 1997).

MMP-13 was not detected in healthy GCF by immunoblot, but MMP-13 activity levels could be detected in both, healthy and disease GCF. MMP-13 expression is highly restricted to those situations in which rapid collagen turnover is required (Rydziel et al., 2000). Despite the fact that MMP-13 levels did not differ among active and inactive sites, the MMP-13 activity was significantly higher in active sites compared with inactive and healthy ones. In accordance with these findings, active sites showed a tendency for MMP-13 levels to increase along with decreasing levels of TIMP-1, displaying a negative correlation. These data suggested that periodontitis might be associated with augmented levels of MMP-13, whereas disease activity might derive, in part, from an unbalance

involving limited MMP-13 inhibition and a higher enzyme activation rate, resulting in increased MMP-13 activity.

In progressive periodontitis, MMP-13 was primarily detected as a band of 56 kDa that could represent a partially activated form derived from the 60 kDa proenzyme (Golub et al., 1997; Hernandez et al., 2006); some faint 48 kDa bands, corresponding to fully active forms, and partially degraded fragments with molecular weight between 35 and 25 kDa (Ilgenli et al., 2006). ProMMP-13 can be activated, among other MMPs, such as MMP-2 and MMP-13 itself, by MMP-14 in the presence of TIMP-1. A first cleavage at the Gly^{35}-Ile^{36} site generates the intermediate fragment, while a second N-terminal Tyr^{85} cleavage generates the fully active enzyme (Knauper et al., 1996) that is highly unstable, generating fragments of low molecular weight (Knauper et al., 1997a).

Similarly to MMP-13 activity, active sites showed increased ICTP levels, assumed to be primarily generated from bone collagen by MMPs (Fuller et al., 2007), when compared with inactive and healthy sites. The addition of MMP-13 to diseased gingival tissue explant cultures resulted in an enhanced proMMP-9 activation rate. Thus, MMP-13 might influence periodontitis progression by contributing to bone catabolism, either directly through its collagenolytic activity or indirectly through the enhancement of MMP-9 activation. Previously, an association between reduced levels of TIMP-1, higher total collagenase and gelatinase activity and the severity of clinical parameters was reported in periodontitis patients, along with significant reductions after periodontal treatment (Pozo et al., 2005). As collagenolytic and gelatinolytic activities are cooperative and sequential during the degradation of native collagen, the enhancement of MMP-9 activation by MMP-13 in

periodontitis might represent a pathogenic mechanism to potentiate and even perpetuate supporting tissue destruction.

Besides sulcular epithelial cells, macrophage-like cells, fibroblasts and plasma cells, MMP-13 is expressed by bone osteoblasts, where it is thought to participate in bone homeostasis (Hernandez et al., 2006; Rydziel et al., 2000). Additionally, MMP-13 is capable of activating proMMP-9 produced by osteoclasts *in vitro* (Rydziel et al., 2000). Therefore, a combination of MMP activity could reflect the release of ICTP fragment to GCF (Golub et al., 1997). The regulatory role of MMP-13 over other MMPs, such as MMP-9, implies that minimal changes in MMP-13 levels or activity can elicit proteolytic downstream cascades that might partially explain periodontitis progression. A previous clinical trial tested a sub-antimicrobial dose doxycycline (SDD) treatment in post-menopausal women exhibiting periodontitis and systemic osteopenia during a two-year follow-up period (Golub et al., 2008). The improvement of clinical parameters in the SDD group was associated with reduced total collagenase activity and a decrease of the bone resorption fragment ICTP. Interestingly, a strong positive correlation between GCF ICTP and total collagenase activity was found, supporting a role for collagenases in bone loss and thereby in disease progression, which can be reflected in GCF.

MMP-8 corresponds to the main collagenase in GCF (Golub et al., 1997), and its active form has previously been associated with the conversion of gingivitis to periodontitis and progression of established periodontitis (Mantyla et al., 2006). In the current study, an increasing trend was seen for the MMP-8 enzyme in active sites versus inactive sites, but it was limited to the active forms from both, neutrophil and mesenchymal isotypes and their

respective percentages of activation, whereas the neutrophil MMP-8 was the predominating isotype (Golub et al., 1997; Golub et al., 2008).

The main source of latent MMP-8 corresponded to neutrophils and could be activated during periodontal inflammation by reactive oxygen species (ROS) and by microbial and host-derived proteases (Sorsa et al., 1992), representing oxidative and proteolytic activation pathways, respectively. Furthermore, ROS can oxidatively inactivate TIMP-1 (Sorsa et al., 2006). Previous *in vitro* results have shown that both, MPO-produced hypochlorous acid and MMP-14-mediated proteolysis can activate proMMP-8 (Holopainen et al., 2003; Saari et al., 1990). Accordingly, we found a significantly strong to moderate positive correlation between total MMP-8 levels, active MMP-8 forms and MPO in both, active and inactive sites at baseline; scaling and root planning (SRP) resulted in significant MPO and MMP-8 reductions in all groups and the loss of MPO/MMP-8 association in treated inactive sites. Nevertheless, the MMP-8/MPO association remained in treated active sites, despite the reduced sample size. Conversely, no association was found between active forms of MMP-8 and MMP-14, suggesting that the oxidative pathway might represent the most important activation mechanism for MMP-8 during periodontitis progression.

Consistent with our findings, it has been reported that repeatedly elevated GCF MMP-8 levels detected during the maintenance phase after SRP was associated with a lack of improvement in clinical parameters (Mantyla et al., 2006) and increased odds of subsequent attachment loss. No association was found between MMP-8 levels and alveolar bone loss in the latter study (Reinhardt et al., 2010). Thus, sustained MMP-8 levels might reflect periodontal breakdown, inflammation or sites at risk of further periodontal loss (Hernandez

et al., 2010; Sorsa et al., 2010). A strong MMP-8/MPO association after periodontal treatment might reflect the persistent activation of MMP-8 and the need for further therapy.

In addition to its classical collagenolytic properties, MMP-8 might influence the immune response through proteolytic processing of non-matrix bioactive molecules, modifying their bioavailability and biological activity (Sorsa et al., 2004; Tester et al., 2007; Van Lint et al., 2005). Since the recent developments of MMP-8 deficient mouse models, new concepts regarding the roles of MMP-8 are emerging. MMP-8 deficient mice have been found to display an altered neutrophil infiltration (Gutierrez-Fernandez et al., 2007; Korpi et al., 2009). Furthermore, recent reports have demonstrated associations between MMP levels and activity with changes in processed forms of cytokines and in their protein levels (Gutierrez-Fernandez et al., 2007; Nilsson et al., 2009).

A previous study had been conducted in a *P. gingivalis*-induced periodontitis MMP-8-deficient mice model, where a more destructive periodontitis phenotype in MMP-8$^{-/-}$ animals was reported. In the current study, we complement these results by demonstrating that MMP-8 KO mice exhibited significantly reduced levels of chemokine LIX/CXCL5 compared with their WT counterparts at the periodontal-oral interface. It is plausible that, given the potent chemoattractant effects of LIX/CXCL5 over PMN (Kebschull et al., 2009), these mice displayed impaired neutrophil infiltration to periodontal tissues, which might explain at some level the extensive alveolar loss observed in these mice. Nevertheless, given the pleiotropic and redundant character of cytokines, further studies are needed. At physiological levels, MMP-8 might be protective for periodontal tissues, whereas pathologically elevated MMP-8 levels or activity might result in excessive periodontal breakdown, explaining the apparently controversial results obtained from the clinical

studies and animal models. Additionally, a significantly higher frequency of detection for the cytokines RANKL and SDF-1/CXCL12 was found in *P gingivalis*-infected mice, irrespectively from the MMP-8 genotype, suggesting that they might play a role in the initiation and/or progression of periodontitis.

In summary, the results of the current study suggest that collagenases MMP-8 and -13 are involved in periodontitis progression and that their pathogenic mechanism is not limited to direct periodontal matrix degradation; rather, they form complex networks involving the immune response, oxidative stress and proteinase cascades. The identification of these networks in biological samples, such as GCF, will contribute to the development of chair side/point of care diagnostic methods to complement clinical practice, to monitor the disease and to identify new targets for pharmacological therapy.

7. CONCLUSIONS

1. Chronic periodontitis subjects show increased GCF volume, total protein concentration and MMP-13 detection compared to healthy controls.

2. Active sites from progressive periodontitis subjects are associated with higher MMP-13 activity and ICTP levels, and reduced detection of the inhibitor TIMP-1 compared with inactive sites. An unbalance between MMP-13 activation vs. inhibition might be involved in soft periodontal tissue and bone catabolism that characterizes active disease.

3. MMP-13 enhances proMMP-9 activation rate in gingival tissue from subjects affected with chronic periodontitis. This MMP interaction might further potentiate proteolytic-mediated tissue destruction during disease.

4. MPO and MMP-8 positive correlation and levels associate with progressive sites at baseline and after periodontal treatment.

5. Experimentally-induced periodontitis is characterized by higher SDF-1/CXCL12 and RANKL detection in periodontitis-affected tissues. Thus, they might play a central role in disease pathogenesis and/or progression.

6. The levels of the neutrophil-chemoattractant LIX/CXCL5 are reduced in infected MMP-8 null mice vs. wild type mice and might result in reduced neutrophil chemotaxis at the oral-periodontal interface and enhanced severity of periodontal disease in MMP-8 null mice.

7. Targeting of MMP-13/ICTP/MMP-9 and MMP-8/MPO interactions in GCF might be potentially useful to screen sites at risk of progression, evaluate response to treatment and pharmacological adjuvant therapies.

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