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PERIODONTITIS AND PERI-IMPLANTITIS BIOMARKERS IN HUMAN ORAL FLUIDS AND THE NULL-ALLELE MOUSE MODEL

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I. Emingil G, Kuula H, Pirilä E, Atilla G, Sorsa T. "Gingival crevicular fluid laminin-5 γ2-chain levels in periodontal disease". J Clin Periodontol 2006; 33: 462-468.

II. Emingil G, Kuula H, Sorsa T, Atilla G. "Gingival crevicular fluid matrix metalloproteinase-25 and -26 levels in periodontal disease". J Periodontol 2006; 77: 664-671.

III. Xu L, Yu Z, Lee HM, Wolff MS, Golub LM, Sorsa T, Kuula H. "Characteristics of collagenase-2 from gingival crevicular fluid and peri-implant sulcular fluid in periodontitis and peri-implantitis patients: pilot study". Acta Odont Scand 2008; 66: 219-224.

IV. Kuula H, Salo T, Pirilä E, Hagström J, Luomanen M, Gutierrez-Fernandes A, Romanos GE, Sorsa T. "Human β-defensin-1 and -2 and matrix metalloproteinase-25 and -26 expression in chronic and aggressive periodontitis and in peri-implantitis". Arch Oral Biol 2008; 53: 175-186.

V. Kuula H, Salo T, Pirilä E, Tuomainen AM, Jauhiainen M, Uitto V-J, Tjäderhane L, Pussinen PJ, Sorsa T. "Local and systemic responses in matrix metalloproteinase 8-deficient mice during *Porphyromonas gingivalis*-induced periodontitis". Infect Immun 2009; 77: 850-859.

ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
ApoA	apolipoprotein A
ABCA1	ATP-binding cassette transporter A 1
BM	basement membrane
BOP	bleeding on probing
C. rectus	*Campylobacter rectus*
CP	chronic periodontitis
CAL	clinical attachment level
COX	cyclo oxygenase
ELISA	enzyme-linked immunosorbent assay
ECM	extracellular matrix
F. nucleatum	*Fusobacterium nucleatum*
AgP	aggressive periodontitis
GCF	gingival crevicular fluid
GPI	glycosylphosphatidylinositol
HDL	high density lipoprotein
hBD	human beta defensin
Ig	immunoglobulin
IL-1β	interleukin-1β
Ln	laminin
LPS	lipopolysaccharide
LDL	low density lipoprotein
MMP	matrix metalloproteinase
$MMP8^{-/-}$	MMP-8 null-allele
MPO	myeloperoxidase
NE	neutrophil elastase
PCR	polymerase chain reaction
PD	probing depth
PI	peri-implantitis
PISF	peri-implant sulcular fluid
P. gingivalis	*Porphyromonas gingivalis*
PMN	polymorphonuclear
P. intermedia	*Prevotella intermedia*
SDD	sub-antimicrobial dose doxycycline
T. forsythensis	*Tannerella forsythensis*
TG	triglyseride
TNF	tumour necrosis factor
VLDL	very low density lipoprotein
WT	wild type

ABSTRACT

Tissue destruction associated with the periodontal disease progression is caused by a cascade of host and microbial factors and proteolytic enzymes. Aberrant laminin-332 (Ln-332), human beta defensin (hBD), and matrix metalloproteinase (MMP) functions have been found in oral inflammatory diseases. The null-allele mouse model appears as the next step in oral disease research. The MMP-8 knock-out mouse model allowed us to clarify the involvement of MMP-8 *in vivo* in oral and related inflammatory diseases where MMP-8 is suggested to play a key role in tissue destruction.

The cleaved Ln-332 γ2-chain species has been implicated in the apical migration of sulcular epithelial cells during the formation of periodontal pockets. We demonstrated that increased Ln-332 fragment levels in gingival crevicular fluid (GCF) are strongly associated with the severity of inflammation in periodontitis. *Porphyromonas gingivalis* trypsin-like proteinase can cleave an intact Ln-332 γ2-chain into smaller fragments and eventually promote the formation of periodontal pockets. hBDs are components of an innate mucosal defense against pathogenic microbes. Our results suggest that *P. gingivalis* trypsin-like proteinase can degrade hBD and thus reduce the innate immune response.

Elevated levels and the increased activity of MMPs have been detected in several pathological tissue-destructive conditions where MMPs are shown to cleave extracellular matrix (ECM) and basement membrane (BM) molecules and to facilitate tissue destruction. Elevated levels of MMP-8 have been reported in many inflammatory diseases. In periodontitis, MMP-8 levels in gingival crevicular fluid (GCF) and in peri-implant sulcular fluid (PISF) are elevated at sites of active inflammation, and the increased levels of MMP-8 are mainly responsible for collagenase activity, which leads to tissue destruction. MMP-25, expressed by neutrophils, is involved in inflammatory diseases and in ECM turnover. MMP-26 can degrade ECM components and serve as an activator of other MMP enzymes. We further confirmed that increased levels and activation of MMP-8, -25, and -26 in GCF, PISF, and inflamed gingival tissue are associated with the severity of periodontal/peri-implant inflammation.

We evaluated the role of MMP-8 in *P. gingivalis*-induced periodontitis by comparing MMP-8 knock-out (*MMP8*$^{-/-}$) and wild-type mice. Surprisingly, MMP-8 significantly attenuated *P. gingivalis*-induced site-specific alveolar bone loss. We also evaluated systemic changes in serum immunoglobulin and lipoprotein profiles among these mouse groups. *P. gingivalis* infection increased HDL/VLDL particle size in the *MMP-8*$^{-/-}$ mice, which is an indicator of lipoprotein responses during systemic inflammation. Serum total LPS and IgG antibody levels were enhanced in both mice groups. *P. gingivalis*-induced periodontitis, especially in *MMP-8*$^{-/-}$ mice, is associated with severe alveolar bone loss and with systemic inflammatory and lipoprotein changes that are likely to be involved in early atherosclerosis.

1. INTRODUCTION

Periodontitis is an infectious disease of hard and soft tissues around the teeth. Bacterial components including enzymes and toxins, together with host responses, ultimately lead to the destruction of hard and soft tissues around the teeth (Kinane et *al.* 1999). Chronic periodontitis can affect a few teeth or the whole dentition in the oral cavity. Chronic periodontitis is strongly associated with bacterial infection and severe inflammation. Aggressive periodontitis, distinct from chronic periodontitis, typically affects younger people and has fewer local triggering factors (Wiebe & Putnins 2000). It usually affects permanent incisors and molars and causes vertical alveolar bone resorption. Even though the aetiopathogenesis of aggressive periodontitis remains unclear, colonisation with several specific Gram-negative anaerobic bacteria, such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis,* is evidently strongly associated with the progression of aggressive periodontitis (Suomalainen et *al.* 1991, Albandar et *al.* 2001, Kinane et *al.* 2001). *P. gingivalis* is able to produce specific proteinases (Sorsa et *al.* 1987, Potempa et *al.* 2000), which can inactivate and destroy hosts' own protective molecules. They are also able to stimulate proteolytic and proinflammatory proteins and enzymes, and disturbances in the normal physiological activity and levels of these molecules lead to periodontal tissue breakdown and to attachment loss of the teeth (Sorsa et *al.* 1992b, 1995, Golub et *al.* 1995).

Dental implants are widely used in dentistry to replace removable dentures among edentulous and partially edentulous patients. Peri-implantitis is an inflammatory disease that affects alveolar bone and soft tissues around implants, eventually leading to loosening of the implant. The aetiopathogenesis of peri-implantitis remains somewhat unclear, but has a similar infectious and inflammatory background to the pathogenesis of periodontitis (Mombelli et *al.* 1987).

Laminin-332 is a glycoprotein essential to the adhesion of epithelial cells, especially in the formation of hemidesmosomes (Gürses et *al.* 1999), and is produced in the oral cavity by gingival epithelial cells (Colognato & Yurchenco 2000). The Ln-332 molecule consists of three different polypeptide chains, of which the γ2-chain is specific to Ln-332 (Colognato & Yurchenco 2000, Aumailley et *al.* 2005). Several matrix metalloproteinases (MMP) can cleave the Ln-332 γ2-chain into smaller fragments (Pirilä et *al.* 2001, 2003). This cleaved Ln-332 stimulates the migration of gingival epithelial cells and is believed to play a central role in the apical migration of sulcular epithelial cells and in the formation of periodontal pockets (Gianelli et *al.* 1997, Pöllänen et *al.* 2003).

The innate immune system recognises invading pathogens, distinguishes pathogenic from non-pathogenic microorganisms, and initiates the co-ordination of the host response to kill pathogens. Human beta defensins (hBDs) are small soluble peptides secreted on epithelial surfaces. They are produced in the epithelial cells of skin, mucosa, the intestine, and in leukocytes. hBDs are part of our own host response against micro-organisms and is why they are also considered "natural antibiotics". hBDs can kill microbes mostly by compromising their membrane integrity (Schröder 1999). hBDs are

also produced in human salivary glands (Mathews et *al.* 1999), though the expression and localisation of hBDs in the oral cavity remains unclear.

MMPs are catalytic proteins, enzymes capable of cleaving almost all extracellular matrix (ECM) and basement membrane (BM) proteins. MMPs are involved in many physiological processes such as bone formation, tooth eruption, and wound healing. On the other hand, MMP expression and activation is disturbed in many pathological conditions such as cancer, oral cysts, and periodontitis (Wahlgren et *al.* 2001, Lopez-Otin et *al.* 2002, Sorsa et *al.* 2004a).

MMP-8 is one of the most efficient enzymes in degrading type I collagen (Sorsa et *al.* 2004). MMP-8 is produced by many different cells in different tissues, but is expressed most dominantly by polymorphonuclear leukocytes (PMN) (Sorsa et *al.* 2006). During inflammation, PMN cells invade from vessels to tissues, where they release MMP-8 (Sorsa et *al.* 2004a). Previous studies have demonstrated that MMP-8 levels are pathologically elevated in human periodontitis and in peri-implantitis (Tervahartiala et *al.* 2000, Kivelä-Rajamäki et *al.* 2003, Mäntylä et *al.* 2003). Still, the role of MMP-8 *in vivo* remains unclear, and recent animal model studies have discovered a protective anti-inflammatory role for MMP-8 in experimental skin and oral cancer as well as in lung inflammation (Balbin et *al.* 2003, Owen et *al.* 2004, Gueders et *al.* 2005, Korpi et *al.* 2008). MMP-25 belongs to the membrane-type MMP subfamily and can degrade gelatine, type IV collagen, and fibronectin in the ECM. MMP-25 is produced mainly in PMN cells (Duanqing 1999), but previous studies have also shown MMP-25 production in breast, lung, intestinal, kidney, and brain tumor cells (Velasco et *al.* 2000, Sun et *al.* 2007, Sohail et *al.* 2008). MMP-26 is produced mainly in the uterus and placenta as well as in various tumor cells (Uria et *al.* 2000). In the ECM, MMP-26 cleaves fibrinogen and some ECM proteins such as fibronectin, gelatine, and type IV collagen. The localisation and expression of both MMP-25 and -26 still remains unclear in periodontitis and in peri-implantitis.

Infections and chronic inflammations increase the risk of developing cardiovascular disease/atherosclerosis and stroke (Armitage 2000, Pussinen et *al.* 2004). The role of inflammation in the pathogenesis of both periodontitis and cardiovascular disease has taken on increased significance (Davé & Van Dyke 2008). Bacterial lipopolysaccharide (LPS), a potent virulence factor among Gram-negative bacteria, is capable of activating macrophages and other inflammatory cells. In the formation of atherosclerotic plaque, LPS-macrophage-inflammatory mediators play a critical role by inducing the release of cytokines, by enhancing platelet aggregation, and by promoting the formation of foam cells (Pussinen et *al.* 2004). Because periodontitis is an infection-associated chronic inflammation, periodontal infections have been considered risk factors for the development of cardiovascular disease and atherosclerosis.

2. REVIEW OF THE LITERATURE

2.1. Healthy periodontium around teeth

Periodontium is a functional unit of tissues that surround the teeth. Normal healthy periodontium consists of gingiva, which covers the jaw bone and is tightly attached to the teeth. Roots anchor the teeth to the alveolar bone via the periodontal ligament, attached to the root cementum (Figure 1). The main function of periodontium is to support, to protect and to provide nourishment to the teeth.
The gingiva must resist the mechanical stimuli of hard food particles as well as large accumulations of plaque bacteria in direct contact with the gingival sulcus. Gingiva can be subdivided into attached, free mariginal, and interdental gingiva. Structurally, gingiva is a combination of epithelial and connective tissues consisting of three distinct phenotypes of epithelium: oral keratinised epithelium facing the oral cavity, the sulcular nonkeratinising epithelium facing the root of the teeth, and the junctional epithelium providing physical attachment to the tooth surface (Locke et *al.* 2008). The gingival tissues constitute the major peripheral defence against microbial infections.

The junctional epithelium is continuous with the sulcular epithelium and, with hemidesmosomes, forms the epithelial attachment to the tooth surface. At its apical termination, it forms the bottom of the gingival sulcus (Bosshardt & Lang 2005). The junctional epithelium protects the periodontal soft tissues lying underneath it from exposure to bacteria and their by-products. The conversion of the junctional epithelium to diseased pocket epithelium is believed to play a key role in the progression of gingivitis to periodontitis (Haffajee & Socransky 1994, Page 2002). Laminin-332, a component of hemidesmosomes, is considered as one of the key factors in the apical migration of epithelial cells and pocket formation in the progression of periodontitis (Pirilä et *al.* 2003). Characteristic of the junctional epithelium is the relatively small number of desmosomes that connect junctional epithelial cells. This is why the intercellular spaces between junctional epithelial cells vary considerably and allow the transmission of white blood cells from blood vessels to the bottom of the gingival sulcus (Schroeder & Listgarten 1997). The junctional epithelium features several defense mechanisms against biofilm-associated bacterial exposure. It can express a wide range of molecules, such as cytokines, chemokines, cytokeratins, and matrix metalloproteinases (Mackenzie et *al.* 1991, Uitto 2002, Smith et *al.* 2004) that can contribute to innate immune defense. In response to bacterial plaque, epithelial cells can produce what are known as natural antimicrobial peptides, human beta defensins (hBDs) that contribute to the innate host defense against microbes (Chung et *al.* 2007, Bosshardt & Lang 2005).

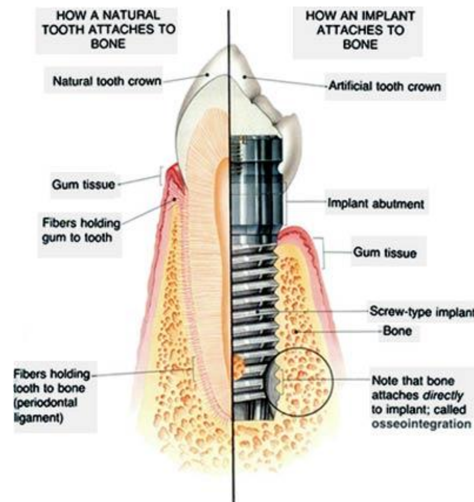
The lamina propria is a thin layer of loose connective tissue which lies beneath the epithelium and, together with the epithelium, constitutes the gingiva. The lamina propria consists mainly of a dense network of collagen fibers, blood, and lymphatic vessels and nerves (Schroeder & Listgarten 1997). The periodontal ligament is a fibrous network that connects the tooth to the alveolar bone or to gingival tissues as gingival fibers. The periodontal ligament fibers consist mainly of type I collagen, and the

fibers connect to the root cementum at the tooth surface (Liu et *al.* 1997). By pressure sensitive receptors within it, it serves a sensory function by allowing the brain to discern the amount of force being placed on a tooth during chewing. The cementum is a calcified tissue that covers the root of the tooth. Its main function is to provide a medium to which the periodontal ligament can attach. It forms a cemento-enamel junction at its coronal top, where it meets the enamel. The alveolar bone is the tooth-bearing bone that contains the tooth sockets and to which the masticatory muscles attach.

2.2. Healthy periodontium around implants

During the past decade, the demand for dental implants, artificial tooth roots, has grown considerably. Dental implants are used to replace removable partial or full dentures in edentulous and partially edentulous patients. Implants and implant-supported prostheses offer greater stability, comfort, and aesthetics than any other removable prostheses. Dental implants are titanium fixtures placed into the jaw bone during surgery. Titanium is the most common biometal used in endosseus dental implants because of its excellent biocompatibility properties in physiological conditions (Sammons et *al.* 2005). The term osseointegration means direct bone contact with an alloplastic metallic implant. The hard and soft tissues surrounding an osseointegrated implant show some similarities to the periodontium around natural dentition (Figure 1) (Myshin et *al.* 2005). The gingiva around dental implants is called peri-implant mucosa, and consists of well-keratinised oral epithelium, sulcular epithelium, and junctional epithelium with underlying connective tissue. Between the implant surface and epithelial cells are hemidesmosomes and the basal lamina (Newman et *al.* 1988). The most significant difference between natural teeth and implants is that implants lack the periodontal ligament. The collagen fibers are unattached and parallel to the implant surface rather than in functional contact from the bone to the cementum. The titanium screw attaches directly to the alveolar bone, which is in direct and tight contact with the implant surface.

Figure 1. Healthy periodontium around a normal tooth and an implant. Reprinted by the permission from University of Connecticut Health Center



2.3. Gingival crevicular fluid

Gingival crevicular fluid (GCF) is either a serum transudate in a healthy, almost inflammation-free gingiva, and more commonly an inflammatory exudate originating from the vessels of the gingival plexus, and is recognised as a part of the gingival defence system. It is rich in leukocytes, particulary polymorphonuclear granylocytes (PMN), which are attracted by chemotactic gradients of bacterial or host origin. It is also rich in different host-derived molecules from blood as well as substances from micro-organisms from bacterial plaque (Lamster & Ahlo 2007). The GCF flow requires vascular permeability induced by initiators of inflammation (Schroeder & Listgarten 1997). It has been demonstrated that GCF flow and volume increases during inflammation (Ozkavaf et *al.* 2000), but increased GCF volume alone cannot be considered a risk factor for periodontitis. There are over 65 different infection-induced enzymes and their inhibitors and regulators (i.e. cytokines, chemokines, and tissue breakdown products found in GCF), some of which appear in Table 1 (Armitage 2004). These components are markers of metabolic changes in the progression of periodontitis and may serve in the diagnostics and investigation of the severity and degree of inflammation (Kinane et *al.* 2003, Sorsa et *al.* 2004a, 2006). In this regard Mäntylä et *al.* (2003, 2006) have investigated a diagnostic tool, a chair-side collagenase-2 test stick, to identify and monitor the course, risk, and treatment of periodontitis. Because GCF is mainly an exudate from blood, it may also indicate systemic diseases. Antibodies can be detected from GCF (Ebersole et *al.* 1990), which has also proved to be a significant source of hepatitis virus in saliva (Maticic et *al.* 2001).

2.4. Peri-implant sulcular fluid

Peri-implant sulcular fluid (PISF) is also an inflammatory exudate originating from the vessels of the gingival plexus. Its composition is similar to GCF, containing host-derived enzymes and their inhibitors, inflammatory mediators and host-response modifiers, and some tissue breakdown products (Table 1) (Armitage 2004). PISF volume, together with increased enzyme activity, has been suggested to be elevated during inflammation (Sorsa et *al.* 2004b), which confirms the diagnostic potential of PISF in peri-implant inflammations.

Table 1. Summary of GCF/PISF components.

Host-derived enzymes and their inhibitors	
Elastase and elastase inhibitors	α_2-macroglobulin, α_1-proteinase inhibitor
Trypsin-like enzymes	
Collagenases	MMP-1, -8, -13
Gelatinases	MMP-2, -9
Tissue inhibitors of MMPs	TIMP-1
Stromyelysins	MMP-3, -10, -11
Myeloperoxidase	
Inflammatory mediators and host-response modifiers	
Cytokines	Interleukins, tumor necrosis factor-α, interferon- α
Antibacterial antibodies	IgGs, IgM, IgA
Substance P	
Prostaglandin E_2	
Acute-phase proteins	Lactoferrin, transferrin, C-reactive protein
Leukotriene B_4	
Tissue breakdown products	
Glycosaminoglycans	Hyaluronic acid, chondroitin sulfates, dermatan sulphate, hydroxyproline, fibronectin fragments
Connective tissue and bone proteins	laminin, osteonectin, osteocalcin, type I collagen peptides, haemoglobin β-chain peptides

2.5. Pathogenesis of periodontitis

Periodontitis is an infectious disease that leads to the destruction of hard and soft tissues surrounding the teeth. Bacterial adhesion to, and colonisation of the teeth surface, biofilm accumulation, and tissue invasion results in clinical symptoms of inflammation, leading to gingivitis. Gingivitis inflammation is confined to the gingiva and is reversible after treatment. If the situation is left without treatment, it may lead to periodontitis where the inflammation extends into deeper tissues, leading to gingival swelling and bleeding. In the late phase of the disease, the supporting collagen of the periodontium degenerates, alveolar bone begins to resorb, and the gingival epithelium migrates along the tooth surface, forming a

periodontal lesion. Intact Ln-332 provides significant adhesive properties between the tooth and the epithelium interface, but after cleavage by MMPs, Ln-332 γ2-chain 40 and 70 kDa fragments can stimulate epithelial cell migration (Gagnoux-Palacios et *al.* 2001, Pirilä et *al.* 2003). This apical migration of epithelial cells is a phenomenon typical of periodontitis, and leads to the formation of a "periodontal pocket" (Pirilä et *al.* 2001, 2003, Pöllänen et *al.* 2003).

Although bacteria are the primary cause of periodontal disease, the expression of microbial pathogenic factors alone may be insufficient to cause periodontitis. Bacterial toxins lead to a host response in which several proteolytic enzymes and a cascade of inflammatory molecules are expressed in the gingiva (Dale 2002). Bacteria produce harmful by-products and enzymes that break extracellular matrices as well as host cell membranes to produce nutrients for their growth. They initiate damage directly or indirectly by triggering host-mediated responses that lead to tissue breakdown (Jain et *al.* 2008). In the progression of periodontitis, neutrophilic granulocytes are the first inflammatory cells in action, followed by antibody and neutrophil axis and leading finally to the activation of monocytes and lymphocytes, which modulate the inflammatory response (Miyasaki 1991, Hart et *al.* 1994). This leads to inflammatory disease activity and hard and soft tissue destruction.

Periodontitis, especially chronic periodontitis, is initiated by an overgrowth of specific, Gram-negative bacterial species (Darveau et *al.* 1997). In human chronic periodontitis, five bacterial species have been found in active lesions: *Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Porphyromonas gingivalis, Tannerella forsythensis, Fusobacterium nucleatum and Campylobacter rectus.* (Moreno et *al.* 1999) *A. actinomycetemcomitans* is also associated with different forms of aggressive periodontitis (Loesche 1993). These organisms have the ability to penetrate the gingival epithelium and to release endotoxins and cytotoxic enzymes and molecules. Pathogens are necessary, but insufficient for disease activity to occur. Factors influencing such activity include the susceptibility of the individual host and the presence of interacting bacterial species (Socranscy & Haffajee 1992). *A. actinomycetemcomitans* produces specific leucotoxin, and the immunologic response of the host to this antigen may be one explanation for the unique pattern of tooth involvement in aggressive periodontitis. *P. gingivalis* in particular produce trypsin-like enzymes which can act as a virulence factor in periodontal inflammation (Sorsa et *al.* 1987).

Although periodontitis is considered a complex interplay of bacterial infection and host response, a variety of factors affect the severity of the disease. Important risk factors include smoking, poorly-controlled diabetes, general health, and poor oral hygiene (Genco 1994). Periodontitis can also be associated with systemic conditions such as metabolic and haematologic disorders, immune system disorders, as well as host genetic factors (Kinane & Marshall 2001). Still, only limited success in periodontal risk assessment has been achieved (Persson 2008).

2.6. Different disease states of periodontium

2.6.1. Classification of periodontal disease

In 1999, an International Workshop for the Classification of Periodontal Disease and Conditions was organised to revise the classification system of periodontal diseases (Armitage 1999). Classification is necessary to scientifically study the aetiology, pathogenesis, and treatment of diseases (Wiebe & Putnins 2000, Armitage 2002). The new classification system is complex and contains numerous subcategories. The major categories of classification are follows: I. gingival disease, II. chronic periodontitis, III. aggressive periodontitis, IV. periodontitis as a manifestation of systemic diseases, V. necrotising periodontal disease, VI. abscesses of the periodontium, VII. periodontitis associated with endodontic lesions, and VIII. development of aquired deformities and conditions. This study I will focus only on the following disease states of the periodontium:

2.6.2 Gingival disease

Plaque-induced gingivitis is the most common type of gingival disease. The clinically normal gingiva in humans always demonstrates a low-grade defence in the presence of dental plaque. The clinical signs of gingivitis are swelling and redness of the gingiva, increased GCF flow, and the breakdown of collagen fibers followed by PMN cell infiltration (Schröder & Listgarten 1997). No hard tissue breakdown or clinical attachment loss occurs in gingivitis.

Other, non-plaque-induced forms of gingivitis also exist and should be classified differently from plaque-associated gingivitis. Systemic diseases, such as diabetes (Ryan et *al.* 2003) and leukemia, as well as endocrine changes (puberty and pregnancy) (Gürsoy et *al.* 2008), different medications, allergic reactions, mucocutaneous infetions, and trauma can cause non-plaque-induced gingivitis. Colonisation by certain bacteria, viruses, and fungi can also cause non-plaque induced gingivitis (Wiebe & Putnins 2000).

2.6.3. Chronic periodontitis

The most common form of periodontitis is the plaque-induced variety, with its characteristic signs of gingival inflammation, apical migration of epithelial cells, and the breakdown of connective tissues and alveolar bone (Armitage 2004). Chronic periodontitis (CP) occur mostly in adults, and subgingival calculus is most commonly found. It is generally a slowly progressing disease, but can also have periods of rapid destruction. Chronic periodontitis can be divided into generalised and localised forms depending on the percentage of sites involved. The severity of CP can also be divided into slight, moderate, and severe CP based on the amount of clinical attachment loss (CAL) (Wiebe & Putnins 2000). As in gingivitis, several risk factors are associated with CP, the most important of which are smoking, traumatic occlusion (Pihlström et *al.* 1986, 2001), type 1 diabetes (Mealey 1999), the presence

of Gram-negative bacteria (A. *actinomycetemcomitans*, *P. gingivalis, T. forsythensis, P. intermedia, F. nucleatum*) (Kinane et *al.* 1999, Moreno et *al.* 1999), some viruses and fungi (Contreras et *al.* 1999), as well as socioeconomic and genetic factors (Beck et *al.* 1997).

2.6.4. Aggressive periodontitis

Aggressive periodontitis (AgP) (localised and generalised) differs from chronic periodontitis by the age of onset of the disease, the presence of only a little of local factors (plaque and calculus), the accelerated progression rate, defect manifestations in the host response, and the composition of subgingival microflora. It typically affects young persons (under 35 years) appearing otherwise healthy, but may also affect older people. It is diagnosed by clinical, radiological, and histological findings that show rapid attachment loss and bone destruction (Wiebe & Putnins 2000). Such patients are usually systemically healthy, and characteristic of AgP is altered neutrophil and immunoglobulin function as well as *A. actinomycetemcomitans* or *P. gingivalis* colonisation or both (Novak & Novak 1996).

2.7. Peri-implantitis

2.7.1. Classification and pathogenesis of peri-implantitis

Peri-implantitis is regarded as an "infection-induced inflammatory process affecting the tissues around an osseointegrated implant in function, resulting in loss of supporting bone" (Albrektsson & Isidor 1994, Sorsa et *al.* 2004b). Although dental implant therapy has been considered to have an excellent prognosis, recent reports on the long-term success of implant therapy have presented surprisingly high prevalence rates of perimucositis and peri-implantitis (Roos-Jansåker et *al.* 2006). A number of risk factors have been identified, including 1) poor oral hygiene, 2) a history of periodontitis, 3) diabetes, and 4) smoking (Klinge et *al.* 2005, Heitz-Mayfield 2008).

Two types of implant failures have been identified and should be considered separately: I) an early implant failure due to occlusal overloading corresponds to the inability to establish osseointegration (Rosenberg et *al.* 2004). Occlusal overload increases the risk for microfractures at the implant-bone interface, which can result in significant marginal bone loss and implant failure (van Steenberghe et *al.* 1999). II) A late implant failure is peri-implantitis, a site-specific inflammatory disease with micro-organisms associated in patterns known from the chronic periodontitis of natural teeth, leading to bone loss and finally to implant failure (Mombelli 2002). The microbial plaque accumulation is considered the most important factor in the pathogenesis of peri-implantitis (Mombelli 1997, Drake et *al.* 1999, Quirynen et *al.* 2001). In the initial stage, plaque accumulation can cause perimucositis, a reversible inflammation of the soft tissues surrounding functional implants (Mombelli & Lang 1998). The adherence of micro-organisms to non-shedding biomaterial surfaces and the successful colonisation of these surfaces are principal factors in biomaterial-associated infections (Lang et *al.* 2000). The peri-implant microflora is established shortly after implant placement (Lee et *al.* 1999), and several studies

have demonstrated that periodontal pathogens, such as *P. intermedia*, can be transmitted from residual teeth to implants (Apse et *al.* 1989, Gerber et *al.* 2006, Kohavi et *al.* 1994, Koka et *al.* 1993, Mombelli 2002). Overall, microbiota found in peri-implant lesions is similar or at least almost similar to that found in periodontal lesions (Listgarten et *al.* 1999, Mombelli et *al.* 1995, Papaioannou et *al.* 1996, van Winkelhoff et *al.* 2000, Quirynen et *al.* 2006, Shibli et *al.* 2007). Distinct from periodontitis, spirochetes are closely linked to peri-implantitis (Rams et *al.* 1983, 1984).

Structurally, the peri-implant epithelium closely resembles the junctional epithelium found around natural teeth (Listgarten et *al.* 1996). The peri-implant epithelium produces inflammatory mediators, and the local host response is biochemically similar to the response observed in periodontitis (Apse et *al.* 1989, Teronen et *al.* 1997, Kivelä-Rajamäki et *al.* 2003, Sorsa et *al.* 2004a, 2004b, 2006). Failing implants affected by peri-implantitis are generally charcterised by:

- The absence of mobility;
- Progressive marginal bone loss resulting in a typical "crater-like" bony defect, while the bottom part of the implant retains perfect osseointegration;
- Signs of infection and inflammation, the infiltration of inflammatory cells, plasma cells and PMNs, and the ulceration and proliferation of the junctional epithelium.

2.8. Matrix metalloproteinases

2.8.1. General characteristics of MMPs

Matrix metalloproteinases (MMP) are catalytic proteins that are expressed throughout the human body and are capable of degrading almost all extracellular matrix (ECM) proteins. They play central roles in many physiological processes such as morphogenesis, bone formation, tooth eruption, and wound healing, but also participate in many pathological conditions and disease progression (Nagase et *al.* 2006). The main function of MMPs is believed to be the degradation and removal of ECM molecules from the tissue, but they may also function as cell receptors. MMP expression and activity are found to be increased in many oral diseases such as oral cancer (Korpi et *al.* 2008), oral cysts (Wahlgren et *al.* 2001), and periodontitis (Sodek & Overall 1992, Sorsa et *al.* 2004a, 2006). Studies using MMP gene knock-out mice have illuminated the crucial role of MMPs not only in maintaining tissue homeostasis, but also in disease progression: MMP-2 and MMP-9 play a significant role in cardiac rupture after myocardial infarction (Hayashidani et *al.* 2003, Matsumura et *al.* 2005) and in aortic aneurysm formation (Longo et *al.* 2002). MMP-9 and MMP-14 are associated with ischemia and repefusion (Romanic et *al.* 2002, Deschamps et *al.* 2005). Studies in atherosclerotic plaque formation indicate that MMP-3 and MMP-9 play a protective role by limiting plaque growth, but MMP-12 instead promotes the expansion of atherosclerotic lesions (Johnson et *al.* 2005). MMP-8 knock-out mouse models have demonstrated the protective role of MMP-8 in cancer progression (Balbin et *al.* 2003, Gutierrez-Fernandes et *al.* 2008, Korpi et *al.* 2008) and in wound healing (Gutierrez-Fernandes et *al.* 2007).

There are 28 MMPs identified in vertebrates, of which 23 are found in humans. Some of the MMPs identified (MMP-4, -5, -6, and -22) appear to be identical to other members. The different MMPs are listed in Table 2. MMPs are classified into six different subfamilies: 1) collagenases, 2) gelatinases, 3) stromelysins, 4) matrilysins, 5) membrane-type MMPs, and 6) others. MMP structure typically consists of a propeptide, a catalytic "metalloproteinase" domain, a hinge region, and a hemopexin domain. Collagenases are the most efficient MMPs to cleave not only collagens I, II, and III, but other ECM molecules and proteins as well. The main substrate of the gelatinase subfamily is gelatine, but both are capable of digesting a number of other ECM molecules. MMP-2 (Gelatinase A) efficiently digests collagens I, II, and III in the same manner as the collagenase subfamily does. Stromelysins digest a number of ECM molecules and participate in proMMP activation.

Table 2. Matrix metalloproteinases and their main substrates.

Enzyme	MMP	Functions and/or main substrates
Collagenases		
Collagenase-1	MMP-1	Collagen types I, II, **III**
Collagenase-2	MMP-8	Collagen types **I**, II, III, VII, VIII, X
Collagenase-3	MMP-13	Collagen type I, II, III, ECM turnover
Collagenase-4	MMP-18 (not found in humans)	Collagen type **I**
Gelatinases		
Gelatinase A	MMP-2	Gelatine, collagens I, II, IV, V, VII, X
Gelatinase B	MMP-9	Gelatine, collagens IV, V, VII, X
Stromelysins		
Stromelysin-1	MMP-3	Collagen IV in BM vessels, fibronectin, proteoglycans
Stromelysin-2	MMP-10	Collagen IV in BM vessels, proteoglycans, matrix glycoproteins
Matrilysins		
Matrilysin-1	MMP-7	Elastin, entactin, fibronectin, collagens IV, V, IX, X
Matrilysin-2	MMP-26	Fibrinogen, fibronectin, type IV collagen
Stromelysin-3	MMP-11	Elastin, collagens IV, V, IX, X
Membrane-type MMPs		
Transmembrane type		
MT1-MMP	MMP-14	Binding and activation of MMP-2, -9, native types of collagens
MT2-MMP	MMP-15	Binding and activation of gelatinases, laminin, fibronectin
MT3-MMP	MMP-16	Binding and activation of gelatinases, laminin, fibronectin
MT5-MMP	MMP-24	Proteoglycan, collagen type I, laminin, fibronectin,
GPI-anchored		
MT4-MMP	MMP-17	Gelatine, fibrin, fibronectin
MT6-MMP	MMP-25	Gelatine, type IV collagen, fibronectin
Others		
Macrophage elastase	MMP-12	Macrophage migration, elastin
-	MMP-19	BM components, ECM molecules, autoantigen in RA
Enamelysin	MMP-20	Amelogenin
-	MMP-21	Gelatine
CA-MMP	MMP-23	Gelatine
-	MMP-27	Gelatine and casein in chickens
Epilysin	MMP-28	Function in wound healing, RA and osteoarthritis

(Modified from Reynolds 1996, Armstrong & Jude 2002, Tsuruda et *al.* 2004, Abraham et *al.* 2005, Nagase et *al.* 2006, Hou et *al.* 2008, Tomlinson et *al.* 2008)

MMP-7 and -26 (matrilysin-1 and -2) differ from other MMPs in that they lack the hemopexin domain and the hinge region. MMP-7 is synthetised mainly by epithelial cells and is capable of processing ECM components and cell surface molecules. Membrane-type MMPs form a group of MMPs that contain a type I transmembrane proteins or glycosylphosphatidylinositol-anchored (GPI-anchored) proteins. They all are activated intracellulary from proforms, and the active forms are expressed on the cell surfaces. The last subfamily of MMPs contains enzymes that cannot be subclassified into any of the MMP subfamilies. MMP-12, also known as metalloelastase or macrophage elastase, is expressed primarly in macrophages, is essential for macrophage migration, and iscapable of digesting several ECM molecules. MMP-19, extensively expressed in leukocytes and in plasma from patients with reumathoid arthritis (RA), is capable of digesting BM components. MMP-20 is a specific enzyme expressed in newly formed enamel and is capable of digesting amelogenin. MMP-21, -23, and -28 feature a similar kind of furin recognition sequence, all of which are activated intracellulary. MMP-27 was first discovered in chicken embryo fibroblasts, but its activity in mammals remains poorly understood.

2.8.2. Activation of MMPs

In normal physiologically healthy tissues low, MMP activity is demonstrated, but the expression of MMPs is transcriptionally controlled by inflammatory cytokines, growth factors, and hormones, and is inhibited by endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (Uitto et *al.* 2003). MMPs are synthetised as pre-proenzymes, from which the signal peptide is then removed and proenzymes are generated. In most cases, this activation requires the removal of prodomain, resulting in active forms of lower molecular weight. Several tissue and plasma proteinases or bacterial proteinases are capable of activating these proMMP forms. Sometimes the prodomain is only partially removed and needs, for example, other active MMPs to complete removal. This activation mechanism is called "stepwise activation" and is typical of MMPs. MT1-MMP, for example can activate proMMP-2 and proMMP-13 on the cell surface (Itoh & Seiki 2005). Another MMP activation system is furin-dependent intracellular activation, and these enzymes either are secreted or act as cell surface-bound active enzymes (Nagase et *al.* 2006). Their activation is regulated by the tissue-specific location of the enzyme, and their inactivation by endogenous inhibitors or proteolysis. MMPs can also be activated by several other factors, such as the treatment of mercurial compounds, SH reagents, detergents, gold compounds, or oxidation (Sorsa et *al.* 2004a).

2.8.3. Inhibition of MMPs

In MMP inhibition, two major types of endogenous inhibitors are α_2-macroglobulin and tissue inhibitors of matrix metalloproteinases (TIMP). α_2-macroglobulin is a plasma glycoprotein that regulates MMP activities in the fluid phase by entrapping the MMP enzyme within the macroglobulin (Strickland et *al.* 1990).

TIMPs are considered the main inhibitors of MMPs in the tissue. Four different TIMPs (TIMP 1-4) are found in vertebrates. TIMPs are capable of inhibiting all MMPs tested thus far but, the inhibitory effect varies between different MMP molecules. For example, TIMP-1 is a poor inhibitor of several MT-MMPs (Stetler-Stevenson 2008). The balance between MMPs and TIMPs is critical for maintaining normal physiological conditions in tissues (Ryan et *al.* 1996). Fedak et *al.* (2004) discovered that a deficiency in TIMP-3 in knock-out mice led to distruption in tissue homeostasis and caused spontaneous left ventricular dilatation, cardiomyocyte hypertrophy, and contractile dysfunction.

The structure of TIMP can be divided into two separate subdomains: N-terminal and C-terminal subdomains from which the N-terminal domain fold is an independent unit with MMP inhibitory activity (Tuuttila et *al.* 1996). The inhibitory effect is accomplished through the co-ordination of the Zn^{2+} of the MMP active site by the TIMP N-terminal site (Brew et *al.* 2000).

2.8.4. MMP-8

MMP-8 (collagenase-2, neutrophil collagenase) is one of the most efficient enzymes in degrading type I collagen. It belongs to the collagenase subfamily and has a characteristic multidomain MMP structure: signal peptide, propeptide, catalytic domain, hinge region, and hemopexin domain. The N-terminal signal peptide directs the preprocollagenase to secretion and is removed from the latent enzyme (Ala-aho & Kähäri 2005). The hemopexin domain is essential in the regulation of substrate specificity and proteolytic activities and also acts as a binding site for TIMPs. A lack of the 9-amino acid sequence in the linker region allows MMP-8 and other collagenases to bind to collagen and cleave the triple helix of fibrillar collagen (Hirose et *al.* 1993).

PMN cells are the first leukocytes to enter the tissue during an inflammatory reaction. MMP-8 is synthetised mainly in maturating PMN in the bone marrow. It is stored in intracellular granules and secreted and released in response to extracellular stimuli (Sorsa et *al.* 2004). The activation of MMP-8 requires oxidative and proteolytic activation mechanisms to act in concert (Sorsa et *al.* 1992). MMP-8 is also expressed by several other cell types such as chondrocytes (Cole et *al.* 1996), rheumatoid synovial fibroblasts and endothelial cells (Hanemaaijer et *al.* 1997), bronchial epithelial cells, and monocytes (Prikk et *al.* 2001). MMP-8 has an affinity for type I collagen, which is the main component in skin and mucosal ECM. MMP-8 levels are demonstrated to be elevated in chronic inflammatory lesions such as in periodontitis-affected GCF and gingival tissue specimens (Mäntylä et *al.* 2003). MMP-8 therefore has the individual ability to accurately assess the prevalence of active disease (Sorsa et *al.* 1999). Studies have demonstrated the involvement of MMP-8 in the regulation of inflammation, cancer progression (Balbin et *al.* 2003), wound healing (Pirilä et *al.* 2007), and in many other physiological and pathological conditions.

2.8.5. MMP-25

MT-MMPs differ from other MMP subfamilies due to their unique functions and structural characteristics. MT-MMPs appear to function by binding to other MMPs and activating them. MT-MMPs exist on cell membranes and are not secreted extracellulary. Rather, they are integrated into cell membranes by a peptide chain consisting of extracellular stalk, transmembrane α-helix, and a small cytoplasmic domain (English et *al.* 2001). Transmembrane-type MT-MMPs (MT-1, -2, -3, -5) have a cytoplasmic tail and are capable of degrading many different ECM components (Table 2). MMP-25, also known as MT6-MMP/leukolysin, is one of the recently identified members in the membrane-type MMP subfamily. It was first identified in leukocytes and found to be specifically expressed by peripheral blood leukocytes (Pei 1999). However, recent studies have demonstrated MMP-25 production in several different tumor cells such as breast, lung, intestinal, kidney, and brain tumor cells (Pei 1999, Velasco et *al.* 2000). MMP-25, together with MMP-17 (MT4-MMP), is subdivided as a GPI-anchored protease because it binds to the cell membrane via a covalent link to glycosylphosphatidylinositol (GPI) (Kojima et *al.* 2000). MMP-25 can be released as a soluble enzyme from the cell surface or secretory vesicles (Kang et *al.* 2001).

Like other MMPs, MMP-25 activity is tightly regulated by TIMPS (English et *al.* 2001). MMP-25 activity is also regulated by the hemopexin-like domain: MMP-25 can interact with other proteins, such as clusterin which is an abundant protein in the body fluid found in tissues and acts as a negative regulator for MMP-25 through this domain (Matsuda et *al.* 2003). MMP-25 is capable of degrading several ECM proteins, but the main substrates for MMP-25 are gelatine, type IV collagen fibrin, and fibronectin (English et *al.* 2001). MMP-25 is thought to play a significant role in the cellular migration and invasion of the ECM and BM. Previous studies have also demonstrated that MMP-25 can act in cancer progression by activating other MMPs, such as proMMP-2, into mature enzymes (Nie et *al.* 2003). It is evidenced, that MT6-MMP is heavily expressed in human cancer and is specifically associated with cancer progression (Sohail et *al.* 2008).

2.8.6. MMP-26

MMP-26 (matrilysin-2) belongs to the matrilysin subfamily of MMPs. It was initially cloned from a human endometrial tumor which is why it is also known as endometase (Park et *al.* 2000). MMP-26, a polypeptide comprising 261 amino acids, is the smallest MMP yet identified. Structurally, it is very similar to MMP-7: it lacks the hinge region and the hemopexin domain and features an unusual "cysteine-switch" motif (Uria & Lopez-Otin 2000, de Coignac et *al.* 2000). MMP-26 specifically cleaves fibrinogen and ECM proteins, including fibronectin, vitronectin, and denatured collagen (Marchenko et *al.* 2001).

In humans, MMP-26 is expressed mainly in the endometrium (Park et *al.* 2000, Isaka et *al.* 2003), placenta, uterus (Uria & Lopez-Otin 2000), and in the gastrointestinal tract (Bister et *al.* 2004); rodents,

however, lack the *mmp*[26] gene (Puente et *al.* 2003). MMP-26 is also specifically expressed in cancer cells of epithelial origin (Uria et *al.* 2000), including lung (Park et *al.* 2000), prostate, and breast cancer cells (Zhao et *al.* 2003, 2004). MMP-26 levels are shown to be elevated in early-stage cancer cells, indicating that MMP-26 could be a marker of early-stage cancers (Ahokas et *al.* 2005). It seems that MMP-26 serves an important and specific function in tumor progression, tissue-remodeling events, and in inflammatory diseases (Park et *al.* 2000, Uria & Lopez-Otin 2000).

2.9. Human beta-defensins

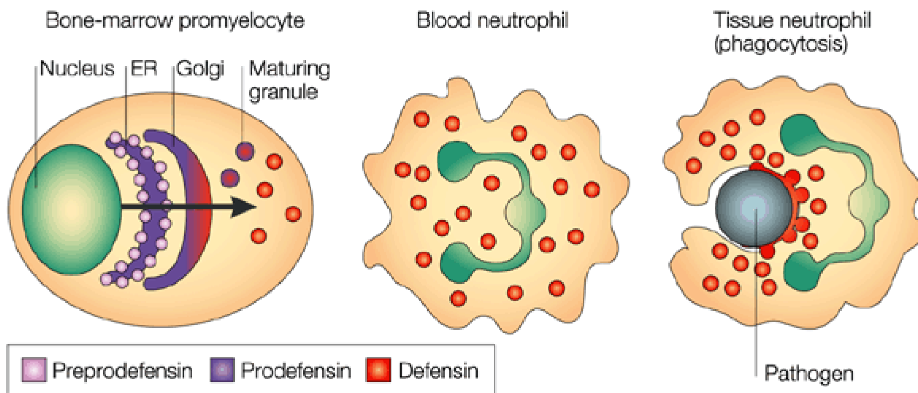
2.9.1. Structure of hBD

In epithelial defence, the epithelium, in addition to its function as a mechanically protective barrier, can produce various cytokines in response to micro-organisms. These cytokines act as inducers of neutrophil and T-lymphocyte chemotaxis. Neutrophils release granules containing several types of microbicidal agents, including human β-defensins (hBD), that are considered "natural antibiotics". hBDs are cationic antimicrobial peptides that belong to the human defensin subfamily. hBDs are abundant in cells and tissues involved in host defence against microbial infections (Ganz 1999), such as in the oral gingival epithelium, and may provide a first line of defence for epithelial and mucosal tissues. In humans, four β-defensins have been characterised in some detail. hBDs are small, comprising fewer than 100 amino acids long polypeptides that consist of a characteristic triple-stranded β-sheet, which forms a distinctive "defensin fold", and a framework of six disulphide-linked cysteines (Ganz 2003).

2.9.2. Expression and activation of hBD

hBD expression is induced by a combination of a specific cell type and tissue environment. hBD expression correlates with differentiation in the epithelium, which is regulated by calcium ions and phospholipase D (Dale et *al.* 2001, Krisnaparkornkit et *al.* 2007). They are expressed mainly in mammalian epithelial cells and phagocytes, and are often present in high concentrations (Ganz 2003). The highest concentrations of defensins are typically found in the specific granules of human PMN (Ganz 1987). Defensins are stored as proforms in azurophilic granules of PMN and undergo processing during their release from the granules. Leukocytes ingest micro-organisms into phagocytic vacuoles to which the defensin-rich granules fuse (Ganz 1987). Paneth cells in the human small intestine also contain defensin-rich secretory granules that are released into crypts of the intestine (Ayabe et *al.* 2000).

Figure 2. Defensins are stored in preproforms of the maturing granules of leukocytes in the bone marrow. During neutrophil differentiation and phagocytosis, proforms are processed into active defensins. Reprinted by the permission from Macmillan Publisher Ltd: Nat Rev Immunol, Ganz T, Sep; 3(9): 710-20, copyright 2003.



hBDs are active against micro-organisms in relatively low concentrations. The most crucial in defensin-mediated antimicrobial activity is the permbealisation of target membranes. Permbealisation inhibits RNA, DNA, and protein synthesis and decreases bacterial viability. hBDs form channels in the negatively charged target membrane with electrical forces that act on the positively charged, cationic defensin molecule. Defensins are more active when the target has a higher negatively charged potential (Wimley et *al.* 1994). Defensins enter the membrane under the influence of cell-generated transmembrane potentials and local electrostatic fields. This is why the tissue environment is crucial to defensin activity; a higher salt and plasma proteins content will inhibit the antimicrobial activity of hBDs by changing the local electrostatic fields (Ganz 2003).

hBDs need to be cleaved with a signal peptidase to release a mature and active peptide (Beckfloff & Diamond 2008). The MMP-7 knock-out mouse model demonstrated a failure to transform intestinal prodefensin into mature defensin, resulting in higher susceptibility to infections (Wilson et *al.* 1999). Moreover, increasing number of bacterial virulence factors have been associated with antagonising the effects of hBDs (Shelburne et *al.* 2005).

In addition to anti-microbial effects, β-defensin expression appears to be upregulated together with pro-inflammatory cytokines in various tissues (Menendez & Finlay 2007). hBDs can stimulate cytokine/chemokine production, keratinocyte migration, and proliferation (Niyonsaba et *al.* 2007), and

function as chemoattractants for immature dendritic cells as well as for T lymphocytes (Yang et *al*. 1999) by binding to specific chemokine receptors (Yang et *al*. 1999) and to mast cells (Niyonsaba et *al*. 2002).

2.9.3. hBD-1

hBD-1 was the first defensin found in the kidney (Bensch et *al*. 1995) and is expressed in numerous mucosal tissues, including the epithelial layers of the vagina, endocervix, uterus, fallopian tubes (Valore et *al*. 1998), lung, and gingival epithelial cells, as well as in the epithelial linings of the urinary and respiratory tracts (Wah et *al*. 2006). hBD-1 is found to be constitutively expressed and plays an important role in innate immunity, but its expression is also modulated by several factors, including inflammatory mediators, micro-organisms, or bacterial LPS. In the oral cavity, hBD-1 is constitutively expressed in gingival tissue (Krisanaprakornkit et *al*. 1998), in the salivary glands (Sahasrabudhe et *al*. 2000), buccal mucosa, palatine tonsil (Chae et *al*. 2001), and tongue (Mathews et *al*. 1999). hBD-1 is believed to play an important role in maintaining oral health. The protective function of hBD-1 depends on continuous peptide expression whereas patients who suffer from hBD-1 deficiency are more susceptible to periodontitis (Pütsep et *al*. 2002).

2.9.4. hBD-2

While hBD-1 is constitutively expressed, hBD-2 is induced in the skin and in other epithelia during inflammation. The most prevalent expression of hBD-2 is observed in the skin and in the gastrointestinal and respiratory tracts (Pazgier et *al*. 2006). The production of hBD-2 is found to require stimulation by proinflammatory mediators of bacterial products for expression (Dale 2002). In the oral cavity, hBD-2 is expressed in the gingival epithelia and mucosa, and the expression is enhanced according to the degree of inflammation (Mathews et *al*. 1999, Vardar-Sengul et *al*. 2007). hBD-2 microbial activity is restricted mainly to Gram-negative bacteria, which are typically detected in periodontal lesions in periodontitis as well as also in other species such as *Eschericia coli, Pseudomonas aeruginosa,* and yeasts such as *Candida albicans* (Harder et *al*. 1997, Sawaki et *al*. 2002). Recent studies have found that viable *A. actinomycetemcomitans* cells induce hBD-2 production in gingival epithelial cells (Kurland et *al*. 2006, Laube et *al*. 2008). Furthermore, *P. gingivalis* proteases have been shown to play a crucial role in the upregulation of hBD-2 (Taguchi & Imai 2006, Chung et *al*. 2004). Bacterial infections can also upregulate the production of hBD-2 in gingival fibroblasts (Rizzo et *al*. 2008), and hBD-2 production by epidermal keratinocytes is enhanced by several cytokines, such as IL-1β (Kanda et *al*. 2008).

2.9.5. Role of hBD-1 and -2 in periodontal disease

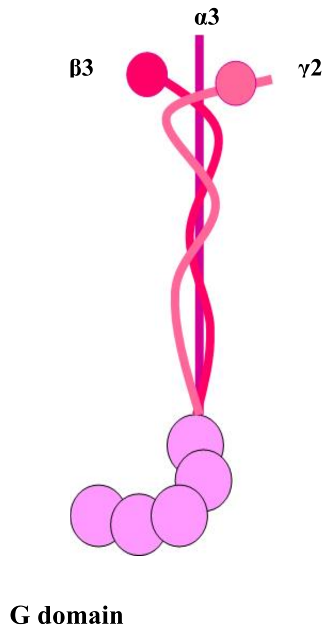
hBDs exhibit antimicrobial activity against oral microbes, including periodontitis-related bacteria, *Candida* sp., and the human papilloma virus (Abiko et *al.* 2007). In addition to the ablility to decimate microbes by causing structural disruption or metabolic injury (Schröder 1999), hBDs also have the demonstrated ability to bind to adhesins of *P. gingivalis,* which may prevent microbial adherence to tissues and induce host inflammatory responses (Dietrich et *al.* 2008). Some periodontal bacteria are able to induce hBDs via interleukin-8, whereas other bacteria found in the human periodontium suppress hBD production. In addition, some periodontal pathogens, such as *A. actimomycetemcomitans,* are more resistant to phagocytosis than are some other bacteria. These findings suggest that various defensins can target different types of bacteria with different cell wall and membrane structures (Ji et *al.* 2007b), and via different activation pathways (Joly et *al.* 2004, Chung & Dale 2008). In human gingiva, hBD-1 and -2 are detected only in differentiating epithelial cells and are predominantly localised in the suprabasal stratified epithelium and in upper epithelial layers consistent with the formation of the stratified epithelial barrier (Dale et *al.* 2001). However, hBDs were undetected in the junctional epithelium (Dale 2002). These findings suggest that hBD-1 and -2 likely serve different roles in the defence against microbes in various regions of the periodontium (Ji et *al.* 2007a, 2007b).

2.10. Laminin-332

2.10.1. Structure of the Ln-332 molecule

Epithelial tissues maintain a stable connection to basement membranes through adhesive interactions between the cells in the basal layer of the epithelium and ECM proteins in the BM. Laminin-332 (Ln-332, previously laminin-5, kalinin, nicein, epiligrin, and ladsin) is a glycoprotein which plays an important role in the adhesion of epithelial cells by forming a core of hemidesmosome structure (Gürses et *al.* 1999). Hemidesmosomes are specific points of connection in epithelia and play an essential role in maintaining firm epithelial adhesion to the basement membrane (Jones et *al.* 1998). Disturbances in Ln-332 expression result in serious mechanobullous skin blistering disorders (Pulkkinen & Uitto 1999). Each laminin chain contains many functional domains, allowing the laminin to interact with various molecules in the ECM (Miyazaki 2006), and based on these chains, each laminin has a specific biological activity. The nomenclature for the laminin family was established by assigning three-digit numbers based on the composition of α-, β-, and γ-chains specific to each laminin isoform (Aumailley et *al.* 2005). Ln-332 consists of three different polypeptide chains, α3-, β3-, and γ2-chains, of which the γ2-chain is specific to Ln-332 molecule (Colognato et *al.* 2000). Three chains are linked by disulfide bonds to form a typical cross-shaped structure (Figure 3). These chains differ significantly in their abilities to interact with other ECM molecules.

Figure 3. Ln-332 structure. The α3-, β3-, and γ2-chains comprise the short arms of the Ln-332 molecule. The G-domain comprises five repeating segments which contain a different kind of binding activity. Adapted by the permission from Macmillan Publishers Ltd: Nature, Marinkovich MP, May; 7(5): 370-80, copyright 2007.



2.10.2. Degradation and processing of Ln-332

Ln-332 interaction with integrins on the cell surface regulates, in addition to epithelial cell adhesion to the BM, normal cellular functions such as proliferation and differentiation (Rousselle & Aumailley 1994). Ln-332 undergoes an extensive and specific proteolysis following secretion. The proteolytic processing of Ln-332 modulates its interactions with surface receptors and other components of ECM, thus regulating Ln-332 function (Sugawara et *al.* 2008). The specific γ2-chain can be proteolysed to smaller fragments, which are believed to induce cell migration (Schenk et *al.* 2003).

Studies demonstrated that some MMP enzymes, such as MMP-2 and -13, are capable of processing an intact Ln-332 γ2-chain into specific, smaller 40 kDa and 70 kDa fragments (Pirilä et *al.* 2003, Wahlgren et *al.* 2003). The cleaved Ln-332 γ2-chain can mediate inflammatory reaction by regulating cell adhesion and the migration and proliferation of epithelial cells and fibroblasts, and by being a chemotactic agent for leukocytes (Gianelli et *al.* 1997).

2.10.3. Role of Ln-332 in periodontal diseases

In the oral cavity, Ln-332 is expressed mainly in epithelial cells. The fragmented Ln-332 γ2-chain stimulates the migration of epithelial cells and, in the formation of periodontal pockets in the progression of periodontitis, is believed to be one of the key factors in the apical migration of epithelial cells (Pöllänen et *al.* 2003). Elevated levels of cleaved Ln-332 γ2-chain fragments in diseased PISF were observed in inflammatory peri-implantitis (Kivelä-Rajamäki et *al.* 2002). Periodontal ligament fibroblasts demonstrate enhanced expression of some other laminin isoforms (Ohshima et *al.* 2006). These findings may indicate that several laminin isofroms are involved in the procession of periodontal inflammatory diseases and tissue remodelling.

2.11. Periodontitis as a risk factor for cardiovascular disease/atherosclerosis

Periodontal disease has been considered a possible risk factor for other systemic diseases such as cardiovascular diseases, stroke, and pre-term infants of low birth weight (Armitage 2000, Pussinen et *al.* 2004, Agueda et *al.* 2008). Research has shown that periodontal disease contributes to an increase in systemic inflammation by promoting an inflammatory and immune systemic response by releasing inflammatory mediators (Persson 2008). Bacteria can destroy periodontal tissues and stimulate host cells to activate a wide range of inflammatory responses (Madianos et *al.* 2005).

Bacterial virulence factors may interact with host cells and lead to inflammatory responses in gingiva. These virulence factors may be involved in modulating inflammatory responses. Bacterial lipopolysaccharide (LPS) can activate macrophages and other cells to synthetise and secrete a wide spectrum of cytokines and chemokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and prostaglandin E_2 as well as MMPs into the blood stream (Pussinen et *al.* 2004). The bacteria associated with periodontitis can produce various bioactive molecules. These inflammation-associated changes cause alterations in the serum lipoproteins towards a more atherogenic profile. Periodontitis causes changes in high-density lipoprotein (HDL) metabolism, which suggests that periodontitis may diminish the antiatherogenic potency of HDL and increase the risk for cardiovascular disease (Pussinen et *al.* 2004). HDL is able to neutralise bacterial LPS in the circulation (Levine et *al.* 1993) and plays a crucial role in reverse cholesterol transport (Fielding et *al.* 1995). HDL acts as an acceptor of cholesterol from cell membranes. This process is facilitated by the interaction of apolipoprotein A-I (ApoA-I) and ATP-binding cassette transporter A 1 (ABCA1) (Fielding & Fielding 1995, Mendez 1997). HDL is essential in cholesterol transport to the liver for exsertion. During inflammation, several changes occur in serum lipoprotein distribution and subclass composition (Pussinen et *al.* 2001). HDL cholesterol concentrations have been shown to decrease during infection (Pussinen et *al.* 2001). In contrast, HDL triglyceride (TG) concentrations seem to increase during inflammation (Newnham & Barter 1990).

Several pathogens, such as *Chlamydia pneumoniae*, *Helicobacter pylori,* cytomegalovirus, and herpesviruses, have been linked to increased risk for coronary heart disease (Armitage 2000). The presence of *A. actinomycetemcomitans* and *P. gingivalis*, two well-known periodontal pathogens, have been detected in atherosclerotic lesions (Kozarov et *al*. 2005). Periodontopathogenic bacteria may contribute to vascular pathology directly, through their cytotoxicity, or indirectly, by inducing inflammation. IgG antibodies to *A. actinomyctemecomitans* and *P. gingivalis* have been associated with coronary heart disease (Pussinen et *al*. 2003). Antibodies to periodontal pathogens increase during periodontitis, and measurements of these serum antibodies could prove useful in determining systemic exposure to periodontopathic pathogens (Pussinen et *al*. 2007).

3. AIMS OF THE STUDY

I. **To investigate the levels, molecular forms, expression and degree of activation of MMP-8, -25, and -26, and of Ln-332 γ2-chain in periodontitis- and peri-implantitis-affected human GCF and PISF**

II. **To investigate the expression and localisation of MMP-25 and -26, and of hBD-1 and -2 in periodontitis- and peri-implantitis-affected human gingival and mucosal tissues**

III. **To clarify the roles of MMP-8 in periodontal inflammation *in vivo* by using the mutant MMP-8 null-allele mouse model**

IV. **To clarify the roles of host defense mechanisms in local and systemic responses to chronic periodontal inflammation**

4. MATERIALS AND METHODS

The detailed methods and reagents used appear in original publications I-V.

4.1. Human study populations and samples

In **study I and II,** GCF samples were collected for the analysis of Ln-332 **(I)**, MMP-25, and MMP-26 **(II)** levels and activities. All consecutive subjects (Table 3) were recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir.

In **study III,** GCF and PISF samples were collected for the analysis of MMP-8 levels and activites. Patients (Table 3) were recruited from the patient pool of the post-graduate clinics of Periodontics & Prosthodontics at the University of Medicine and Dentistry, New Jersey (UMDNJ).

In **study IV,** GCF, PISF, and gingival/mucosal tissue samples were collected to analyse MMP-25, MMP-26, hBD-1, and hBD-2 immunolocalisation, levels, and activites. Patients (Table 3) were recruited from the patient pool of the post-graduate clinics at the Institute of Dentistry, University of Helsinki, and at a private practice in Helsinki, Finland.

All the subjects included in **studies I-IV** underwent a clinical periodontal examination to determine their clinical periodontal status. All measurements were performed at six sites per tooth for the entire mouth. Clinical diagnosis of the CP patients was based on the diagnostic criteria of Baer (1971). The CP group suffered moderate to severe alveolar bone loss. Characteristic of the G-AgP patients were a generalised pattern of severe destruction of eight or more teeth, of which at least three were not central incisors or first molars. Characteristics of periodontal, peri-implant, gingivitis, and healthy sites studied are listed in Table 4.

Table 3. Patient groups (I-IV).

Number of patients (F/M):	**Study I**	**Study II**	**Study III**	**Study IV**
Chronic periodontitis	29 (13/16)	29 (9/20)	10 (5/5)	6 (2/4)
Aggressive periodontitis	18 (9/9)	35 (19/16)	-	5 (1/4)
Gingivitis	20 (8/12)	20 (8/12)	5 (4/1)	-
Healthy	20 (10/10)	21 (11/10)	9 (4/5)	6 (1/5)
Peri-implantitis	-	-	5 (3/2)	11 (9/2)
Total number of patients	**87 (40/47)**	**105 (47/58)**	**29 (16/13)**	**28 (13/15)**

Complete medical and dental histories were taken from all subjects. All subjects were 1) healthy, 2) non-smokers, 3) had at least 20 teeth in their mouth, 4) no history of systemic disease, 5) and had received no antibiotics or other medication or periodontal treatment within the previous four months. Individuals with a medical condition requiring pre-medication prior to dental procedures/visits or currently using antibiotics for any purpose, host modulating agents, or NSAIDs daily, or suffering from any other disease of the oral mucosal or alveolar tissues, pregnant/nursing women, and immune-compromised individuals were excluded.

Approval for the studies was provided by the Ethics Committee of the Institute of Dentistry, University of Helsinki, Helsinki, Finland; the Ethics Committees of the School of Dental Medicine, Stony Brook University, Stony Brook, New York, USA and of the UMDNJ; and the Ethics Committee of the School of Dentistry, Ege University, İzmir, Turkey. The purpose of the study was throughly explained to and informed consent was obtained from all subjects before participating the study.

Table 4. Characteristics of periodontal, peri-implant, gingivitis, and healthy sites studied (I-IV)

	Chronic periodontitis	**Generalised aggressive periodontitis**	**Gingivitis**	**Healthy**	**Peri-implantitis**
Retentive calculus (Y/N)	Y	Y/N	N	N	N
BOP (Y/N)	Y	Y	Y	N	Y
Visible plaque (Y/N)	Y	Y/N	Y	N	Y
Pocket depth (mm)	≥ 4	≥ 5	< 3	< 3	4-6
Tooth/implant mobility (0-2)	0-2	0-2	0	0	0
Gingival/mucosal inflammation (Y/N)	Y	Y/N	Y	N	Y
Radiographic bone loss (Y/N)	Y	Y	N	N	Y

4.1.1. GCF, PISF and gingival/mucosal tissue samples

GCF and PISF samples were collected from sample sites with precut sterile strips of filter paper. Prior to GCF/PISF sampling, the crowns of the teeth/implants were cleaned to remove supragingival plaque, and the strips were inserted into an isolate air-dried gingival/mucosal sulcus or pocket until slight resistance was felt. The samples were then immediately placed into microfuge tubes on ice and transferred to -40 to -80°C for storage until processed for analysis of collagenase activity and Western immunoblotting. In **studies I-III,** the volume of GCF/PISF collected was determined with a

Periotoron 6000/8000, the readings of which were converted to an actual volume (*μl*) by reference to the standard.

In **study IV,** gingival tissue samples were collected from deep (> 6 mm) periodontitis pockets by a surgical incision longitudinally targeting the sulcular (not oral) epithelium and the bottom of the pocket. Gingival biopsies from the attached tissues around implants were obtained with the usual external gingivectomy surgical procedure. The gingival and peri-implant tissue specimens were fixed in 10% formalin and processed in paraffin blocks for immunohistochemical and immunofluorescence analysis.

4.1.2. Clinical measurements (I-III)

In **studies I-III,** pocket depth (PD), clinical attachment level (CAL), bleeding and probing (BOP), and plaque index (PI) were recorded. In all studies, clinical measurements were made with a manual probe (Table 4).

4.2. Western immunoblotting (I-IV)

The presence and molecular forms of Ln-332 from GCF and the cleavage of Ln-332 by *P. gingivalis* trypsin-like proteinase **(I)**, MMP-8 **(III)**, MMP-25 and -26 from GCF and PISF **(II, IV)**, and hBD-1 and hBD-2 from GCF and PISF and dissolved human plasmacytoma cells and macrophage lysates **(IV)** were analysed using the Western immunoblot method. The sources of the antibodies appear in the original publications I-IV. The samples were boiled in non-reducing Laemmli's buffer, and proteins were separated by gel-electrophoresis, electrotransferred to nitrocellulose membranes, and incubated in 5% milk powder. The membranes were first incubated with specific primary antibodies at room temperature overnight followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Proteins were detected by incubation with an enhanced chemiluminesence technique and quantitated with a Bio-Rad Model GS-700 Imagining Densitometer using the Analyst™ programme with correction for background values (Sorsa et *al.* 1994).

4.3. Ln-332, hBD-1, and hBD-2 cleavages by *P. gingivalis* trypsin-like proteinase and MMP-26 (I, IV)

The *P. gingivalis* trypsin-like proteinase was purified as described by Sorsa et *al.* (1987). Intact Ln-332 *γ*2-chain **(I)** was incubated with buffer and partially purified 1 *μ*g *P. gingivalis* trypsin-like proteinase for 1 h and 5 h at 37°C. Resulting cleavage products were analysed with Western immunoblot. Ln-332 *γ*2-chain concentration was calculated by dividing the amount of Ln-332 *γ*2-chain (densitometric unit) by the volume of the sample.
Synthetic hBD-1 and -2 **(IV)** were incubated for 24 h and 48 h at 37°C with autoactive MMP-26 and partially purified trypsin-like proteinase from *P. gingivalis*; 1.5 mM β-mercaptoethanol was included in

incubations with *P. gingivalis* proteinase, but not in those with MMP-26. Reactions were analysed on SDS-PAGE and stained with a Silver Staining Kit using the manufacturer's instructions.

4.4. Collagenase activity analysis (III)

In **study III,** collagenase activity was measured with DNP-octapeptide containing gly-ileu susceptible peptide bond as a substrate. GCF extract or enzyme was incubated with 1 mmol substrate solution for 18 h at 37°C. The reaction mixture was stopped by adding the stop-solution, the samples were vortexed and centrifuged for 5 min at 10 000 rpm. An aliquot was injected into the HPLC for analysis, and the percentage of lysis was calculated (Golub et *al.* 1990).

4.5. Cell cultures (IV)

In **study IV,** human myeloma cell line RPMI 8226 (ATCC no CCL 155) was cultured in RPMI 1640 medium, supplemented with newborn calf serum, lactate glutamate, and penicillin streptomycin, subcultured into reptacles, and cultured in serum-free culture medium for 24 h. Culture medium was centrifuged to separate cells from the medium, and about 10 000 cells were counted for Western blot analysis (Wahlgren et *al.* 2001). Total proteins were isolated from dissolved cells using the Trizol® Reagent isolation method. Macrophage/monocyte cells were grown in RPMI 1640 medium supplemented with fetal calf serum, sodium pyruvate, HEPES, penicillin, and streptomycin (Bellosta et *al.* 2001). Cells were lysed on ice for 30 min in RIPA buffer containing complete protease inhibitor cocktail and centrifuged; the soluble fraction was then collected and analysed using Western immunoblotting as described above.

4.6. Immunostainings (IV-V)

4.6.1. Immunohistochemistry (IV-V)

Immunohistological stainings were performed using standard procedures as well as commercial and non-commercial antibodies as described in detail in **studies IV-V**. Paraffin-embedded tissue specimens were deparaffinised, and immunostainings for lambda plasma cell-marker **(IV)**, CD68 (clone KP1) macrophage-marker **(IV)**, MMP-9 **(V)**, MMP-13 **(V)**, MMP-25 **(IV-V)**, MMP-26 **(IV)**, COX-1 **(IV)**, COX-2 **(IV)**, myeloperoxidase **(V)**, laminin-332 **(V)**, neutrophil elastase **(V)**, IL-1β **(V)**, and TNF-α **(V)** were performed (Lindy et *al.* 1997, Pirilä et *al.* 2001) with monoclonal or polyclonal Vecstain® *Elite* mouse, rabbit, or goat ABC kits. Sections were pretreated, endogenous peroxidase activity was blocked with incubation in 0.6% H_2O_2 in methanol. Samples were blocked with specific normal serum and incubated with specific monoclonal or polyclonal primary overnight. The control sections were incubated with non-immune rabbit or goat serum and showed no stainings. The samples were then incubated with secondary antibodies, and thereafter with Avidin-Biotin enzyme complex. Sections were

stained using 3-amino-9-ethylcarbazole (AEC) as achromogen, counterstained with Mayer's hematoxylin, and mounted in DAKO's glycergel.

4.6.2. Immunofluorescent staining (IV)

In **study IV** gingival and peri-implant tissue specimens were deparaffinised and pretreated with 0.4% pepsin, fixed in a series of ethanol followed in cold acetone, and air-dried. Samples were blocked with normal goat serum and incubated with rabbit polyclonal anti-hBD-1 or anti-hBD-2 antibody overnight. The control sections were incubated with non-immune rabbit serum. The samples were subsequently incubated with fluorescein isothiocyanate-conjugated secondary antibody, the nuclei of the cells were stained with 4',6-Diamidino-2-Phenyl-Indole, and mounted in DAKO's glycergel.

4.6.3. Evaluation of immmunostainings (IV-V)

Immunohistochemical **(V)** and immunofluorescent **(IV)** stainings were evaluated with light or fluorescent microscopy using the AnalySIS programme under an Olympus BX61 microscope. In **study IV,** any intensity (if present) in the immunofluorescent stainings was semi-quantified into three degrees (-, none; +, weak; and ++, moderate/strong positive staining) (Prikk et *al.* 2001, Wahlgren et *al.* 2003). In **study V,** immunohistochemical stainings were semi-quantified into five degrees (0, none; 1, very mild; 2, mild; 3, moderate; and 4, strong positive staining) (Prikk et *al.* 2001).

4.7. RT-PCR (IV)

RT-PCR was performed using 3 g of total RNA as a template and random hexamers as initial primers (Moilanen et *al.* 2002). PCR for MMP-25, MMP-26, hBD-1, and hBD-2 was carried out under the following conditions: 10 min at 95ºC, 35 cycles of 45 s at 95ºC, 45 s at annealing temperature, 45 s at 72ºC, followed by 15 min at 72ºC. The PCR-reaction was carried out in a 25 μl reaction consisting of 1 x AmpliTaq Gold® PCR-buffer, 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 1 μM of each primer, 2.5 U AmpliTaq Gold® DNA polymerase and 5 μl of RT-PCR-reaction as template or 4 μl of PCR-product for nested PCR. β-actin served as an endogenous control, and PCR was carried out using commercially purchased β-actin primers, yielding a 294 bp DNA fragment. The primers used are described in detail in **study IV.**

The annealing temperatures were as follows: MMP-25 and MMP-26 PCR 54ºC, MMP-25 and MMP-26 nested PCR 62ºC, hBD-1 54ºC and hBD-2 60ºC. PCR-fragments were analysed on a 1.5% agarose gel containing 1 μg/ml ethidium bromide prepared in a Tris-acetate-buffer, and DNA-fragments were visualised and photographed on a regular UV-table.

4.8. Animal model of periodontitis (V)

The Committee of Animal Experimentation of the University of Oulu, Oulu, Finland, approved the study protocol. *MMP8*$^{-/-}$ mice of a mixed C57BL/6J/129 background (Balbin et *al.* 2003) were raised, and wild-type littermates served as controls. The mice were bred and maintained in the experimental animal facilities at the University of Oulu, Finland.

4.9. Bacterial strains and culturing (V)

A *P. gingivalis* (ATCC 33277) strain was cultured on Brucella agar plates and incubated in anaerobic jars filled with mixed gas at 37°C for three days. Single bacterial colonies were transferred to new Brucella agar plates and incubated anaerobically at 37°C for two days. Bacterial cells were harvested to sterile 3% CMC (carboxymethyl cellulose medium), and the density of the culture was determined spectrophotometrically at 492 nm to achieve a concentration of $\approx 2 \times 10^9$ CFU/ml.

4.10. Induction of experimental periodontitis (V)

Experimental periodontitis was induced as described by Chang et *al.* (1994). The mice received a mixture of antibiotics (20 mg kanamycin and 20 mg amphicillin) in sterile water twice daily for three days to eliminate the native flora and to promote the subsequent colonisation of *P. gingivalis* in the oral cavity. The antibiotics were allowed to clear from the system for four days. Oral cavities were inoculated with a 3% CMC suspension containing fresh, viable *P. gingivalis* cells twice daily for three days. The control animals received saline and served as negative controls for infection. Thirty days after the last inoculation, blood samples were collected and the animals were killed by cervical dislocalisation. The skulls were dissected, hemisected, and collected for alveolar bone loss measurements and immunohistochemical analysis.

4.11. Analysis of alveolar bone loss (V)

The hemisected skulls were fixed in 10% formalin, decalcified in 12.5% EDTA, and embedded in paraffin. Serial sections were cut and the sections best representing the longitudinal cutting of the first and second molars from the maxillae and mandible were chosen and stained with routine hematoxylin and eosin for histological analysis of alveolar bone loss. The depth of the bony pocket was measured as the vertical distance from the cemento-enamel junction to the alveolar ridge by using AnalySIS programme under Olympus BX61 light microscope. Each site was measured three times at random.

4.12. Serum determinations (V)

Blood samples were collected under CO_2 anaesthesia using cardiac puncture from each animal before termination; the sera were then separated, frozen in liquid nitrogen, and stored at -70°C until serum lipid and inflammation marker analysis.

4.12.1. Lipoprotein total concentrations

Serum samples were analysed for total concentrations of cholesterol, triglycerides, apolipoprotein A-I (van Haperen et *al.* 2000), and LPS.

4.12.2. Multiserotype-ELISA

Serum IgA- and IgG-class antibody levels against *P. gingivalis* were determined using multiserotype-ELISA. Formalin-killed whole cells of three serotypes (ATCC 33277, W50, and OMGS 434) of *P. gingivalis* served as antigens (Pussinen et *al.* 2002). Two dilutions (1:100 and 1:200) of each serum (stored at -70°C) in duplicate were used for the measurements, and the results consisting of mean absorbances were calculated.

4.12.3. Lipoprotein profiles

To obtain lipoprotein profiles, serum samples from each group were pooled (8-12 mice/pool). Pool aliquots of 200 µl were applied on a Superose 6HR size-exclusion chromatography column previously equilibrated with PBS at a flow rate of 0.5 ml/min in PBS, and 0.5 ml fractions were collected. The fractions were analysed for cholesterol, triglyceride, and apoA-I concentrations.

4.13. Statistics (I-V)

In **studies I-II** comparisons were performed using the Kruskal-Wallis test. In case of significant differences, post-hoc two-group comparisons were assessed with Bonferroni-corrected Mann-Whitney U tests. Spearman rank correlation analysis was used to analyse the correlations between GCF Ln-5 *γ*2-chain **(I)**, MMP-25, and MMP-26 **(II)** levels and clinical parameters. All data analysis was performed using a statistical package. Differences were accepted as statistically significant at $p < 0.05$.

In **study III** mean values for relative gingival index, probing depth, gingival crevicular fluid flow rate, and collagenase activities were calculated per sample site (Reinhardt et *al.* 2007). Analysis of variance (ANOVA) was performed to analyze a general linear sample group. Dunn's method was used for all pairwise multiple comparisons. Differences were accepted as statistically significant at $p < 0.05$.

In **study V** using ANOVA variance analysis, alveolar bone loss and serum lipoprotein profiles between the four groups studied were compared. In case of significant differences, Duncan's test was used to perform post-hoc multiple comparisons. In immunohistochemical analysis, post-hoc multiple group comparisons were performed using the Mann-Whitney U test. Differences were accepted as statistically significant at $p < 0.05$.

5. RESULTS

5.1. Clinical measurements (I-III)

In **studies I** and **II** the G-AgP and CP groups had similar mean PD and CAL scores which were significantly higher than those of the gingivitis and healthy groups ($p < 0.05$). The G-AgP, CP, and gingivitis groups had a significantly higher percentage of sites with BOP and plaque than did the healthy group ($p < 0.05$).

In **study I,** Ln-332 γ2-chain 40 kDa and 70 kDa fragment levels correlated positively with both CAL and the percentage of sites with bleeding ($p < 0.05$), but only slightly with PD ($p = 0.05$). GCF volume also correlated with total levels of Ln-332 γ2-chain 40 kDa and 70 kDa fragments ($p < 0.05$).

In **study II,** there was correlation between GCF MMP-25 immunoreactivity with the percentage of sites with bleeding ($p < 0.05$). GCF MMP-26 immunoreactivity correlated positively with all clinical periodontal parameters ($p < 0.05$). There was also a strong correlation between GCF MMP-25 and MMP-26 immunoreactivities ($p < 0.05$).

In **study III,** the clinical and biochemical profiles of CP, and PI showed significant increase ($p < 0.05$) over those of healthy gingiva/mucosa in GCF/PISF flow, PD, and collagenase activity (expressed per site or per µl). The CALs of moderate and severe CP teeth were higher than those of healthy teeth ($p < 0.05$). GCF and PISF analyses showed higher GCF/PISF flows (µl per 10 s) in gingivitis, moderate and severe CP, as well as in PI than the volume reading of GCF/PISF collected from their corresponding healthy controls ($p < 0.05$).

5.2. Levels and molecular forms of Ln-332 in GCF (I)

In **study I,** GCF Ln-332 γ2-chain 40 kDa and 70 kDa fragment levels were significantly elevated in the CP group when comparing the CP group to the gingivitis and healthy groups ($p < 0.05$) but only a tendency of elevated GCF Ln-332 γ2-chain 40 kDa and 70 kDa fragment levels were found between the CP and G-AgP groups. When the data were expressed as a concentration, significant differences in the GCF Ln-332 γ2-chain concentrations were found between the groups studied. Among the groups, the healthy group had the highest GCF Ln-332 γ2-chain 40 kDa and 70 kDa fragment concentration compared with the diseased groups, and the difference was significant when compared with the G-AgP group ($p < 0.05$).

5.3. Levels, activation, and molecular forms of MMPs in GCF and PISF (II-IV)

5.3.1. MMP-8 (III)

In **study III,** the level of MMP-8 immunoreactivity in PISF and GCF was enhanced with the increased clinical severity of both peri-implantitis and periodontal disease. Moreover, the degree of PMN-type MMP-8 activation was clearly enhanced along with the increased clinical severity of both peri-implantitis and periodontal disease. Diseased PISF contained more activated and complexed MMP-8 species than did diseased GCF. Fibroblast-type MMP-8 could be detected in partially activated forms only in GCF and PISF from severe CP and PI. The collagenase activity per site and per µl was higher in moderate and severe CP and in PI than in gingivitis and healthy sites ($p < 0.05$), and was the highest in PI sites.

5.3.2. MMP-25 (II, IV)

In **studies II** and **IV**, western immunoblotting revealed 57-kDa soluble pro-MMP-25, 45- to 47-kDa proactive MMP-25, and 29 kDa fragments, especially in G-AgP, CP GCF, and diseased PISF, whereas only weak or undetectable immunoreactivities were found in the gingivitis GCF and in the healthy GCF/PISF. The G-AgP and CP groups tended to have higher GCF MMP-25 levels compared to the gingivitis group. No significant difference was found between the gingivitis and healthy GCF groups. When the data were expressed as concentrations, no significant differences in GCF MMP-25 concentrations were found between the study groups.
In human plasmacytoma cell lysates, 29 kDa and 57 kDa immunoreactive MMP-25 species were detected, but no corresponding MMP-25 species could be found in macrophage/monocytic cell lysates.

5.3.3. MMP-26 (II, IV)

Western immunoblotting revealed that enhanced levels of 30-kDa pro-MMP-26 and active 19-kDa MMP-26 immunoreactivities were observed, especially in G-AgP, CP GCF, and diseased PISF relative to the lower-level immunoreactivities of MMP-26 in gingivitis and healthy GCF/PISF. The G-AgP and CP groups had significantly higher GCF MMP-26 levels than did the gingivitis and healthy GCF groups ($p < 0.05$). No significant difference was found between the gingivitis and healthy GCF groups. When the data were expressed as concentrations, no significant differences in GCF MMP-26 concentrations were found between the study groups.

5.4. Cleavages of Ln-332, hBD-1, and -2 by *P. gingivalis* trypsin-like proteinase or MMP-26 (I, IV)

Study I confirmed that *P. gingivalis* trypsin-like proteinase produced multiple-size fragments of lower molecular weight and short peptides from mature, unprocessed 100 kDa and 140 kDa Ln-332 γ2-chain

species, thus differing clearly from human MMPs, which cleave Ln-332 γ2-chain into 40 kDa and 70 kDa fragments.
P. gingivalis trypsin-like proteinase, but not human MMP-26 (**IV**), degraded hBD-1 and -2 into species of lower molecular size.

5.5. Levels of MMPs in human periodontitis- and peri-implantitis-affected gingival/mucosal tissue (IV)

MMP-25 immunostaining was observed mainly in plasma cells in gingival tissue specimens of CP and G-AgP patients, but MMP-25 could also be found in PMN cells in PI sections.
MMP-26 was predominantly located in the basement membrane zone in the CP and PI samples. In the G-AgP samples, only weak MMP-26 expression was found in the epithelial basement membrane zone. In non-inflamed gingival tissue samples, MMP-25 or MMP-26 stainings were undetectable.

5.6. Levels of hBDs in human periodontitis- and peri-implantitis-affected gingival/mucosal tissue (IV)

hBD-1 immunoreactivity was present in the oral and sulcular epithelial cells of the CP and G-AgP samples, but weaker in the G-AgP samples. hBD-1 was also expressed in the endothelial cells and in the perivascular BMZ of the CP, G-AgP, and PI samples. In the healthy gingival samples, hBD-1 was detected in the oral and sulcular epithelial cells and in the perivascular basement membrane zone, although to a lesser extent than in the inflamed gingival tissues.
Weak expression of hBD-2 was detected in vessels of the CP, G-AgP, and PI samples, and the perivascular BMZ was weakly positive in the G-AgP and PI samples. Healthy gingival samples showed no detectable expression of hBD-2.

5.7. MMP-25, -26, hBD-1, and -2 mRNA expression in human gingival/mucosal tissues (IV)

MMP-25 and -26 mRNA were expressed at a low level in the inflamed periodontitis and peri-implantitis gingival/mucosal tissues as well as in the healthy groups. hBD-1 and -2 also demonstrated low-level mRNA expression in the healthy and diseased groups.

5.8. Alveolar bone loss in experimental mouse periodontitis (V)

In **study V**, the results of *P. gingivalis*-induced experimental periodontitis demonstrated enhanced alveolar bone loss in the *MMP8*$^{-/-}$ + *P. gingivalis* group relative to the *P. gingivalis* -infected WT group. When mandibular sites were compared, the difference was significant ($p < 0.05$). Bone loss varied considerably between periodontal sites. Both *P. gingivalis* -infected mouse groups exhibited more severe bone loss than did the non-infected control groups ($p < 0.05$). No statistical difference in alveolar bone loss was found between the two control groups (uninfected).

5.9. Levels of inflammation-induced molecules in mouse gingival tissues (V)

The immunohistochemical staining in *MMP8*$^{-/-}$ and WT gingival tissues demonstrated significantly ($p < 0.05$) higher neutrophil elastase levels in both infected groups than in their their uninfected control groups.
MMP-9, TNF-α, and COX-1 exhibited significantly ($p < 0.05$) higher expression levels in the *MMP8*$^{-/-}$ + *P. gingivalis* group than in the *MMP8*$^{-/-}$ uninfected group. A tendency of higher MMP-9 and COX-1 expressions were found between the *MMP8*$^{-/-}$ + *P. gingivalis* group and the WT + *P. gingivalis* group.
Ln-332 expression was the highest among the WT + *P. gingivalis* group, and compared to the WT control group, the difference was significant ($p < 0.05$).

5.10. Mouse serum lipid determinations (V)

5.10.1. Serum total cholesterol, trigyceride, and apoA-I concentrations

Serum lipid and lipoprotein profiles revealed that in the infected *MMP8*$^{-/-}$ mice, the total cholesterol concentration was clearly lower in the *P. gingivalis*-infected mice than in the uninfected *MMP8*$^{-/-}$ mice. The serum triglyceride concentration was higher in the infected *MMP8*$^{-/-}$ mice than in the control group ($p < 0.05$). ApoA-I levels were lower in both *MMP8*$^{-/-}$ groups than in both WT groups ($p < 0.05$).

5.10.2. Serum LPS, IgA- and IgG-levels

Serum LPS concentrations were clearly higher in the *P. gingivalis*-infected mice than in the uninfected mice. The difference between the infected *MMP8*$^{-/-}$ and the uninfected *MMP8*$^{-/-}$ group was significant ($p < 0.05$).
Serum *P. gingivalis* IgG levels were higher in both the WT and *MMP8*$^{-/-}$ bacteria-treated mice than in the controls; the difference between the infected and uninfected WT groups was significant ($p = 0.05$), whereas the *P. gingivalis* IgA-class antibodies were undetectable.

5.10.3. Lipoprotein profiles

Serum lipoprotein analysis demonstrated changes in the distribution of high-density (HDL) and very low-density (LDL) lipoprotein particles; unlike the WT mice, the *MMP8*$^{-/-}$ mice underwent a shift towards a smaller HDL/VLDL particle size: an obvious rearrangement in the distribution of HDL/VLDL subclasses in *MMP8*$^{-/-}$ mice. *P. gingivalis* infection increased HDL/VLDL particle size in the *MMP8*$^{-/-}$ mice, which is an indicator of lipoprotein responses during systemic inflammation. Among the WT mice, *P. gingivalis* infection did not influence the elution position of HDL, thus suggesting no significant changes in HDL particle size. No significant changes were observed in the elution position of LDL particles between the mouse groups.

6. DISCUSSION

6.1. Ln-332 degree and activation in GCF and immunolocalisation in periodontal tissue

In the progression of periodontitis, the cleavage of Ln-332 γ2-chain into smaller fragments can reportedly stimulate epithelial cell migration (Pirilä et *al.* 2001, 2003) resulting in periodontal pocket formation. Several MMPs are capable of cleaving intact Ln-332 into smaller fragments (Wahlgren et *al.* 2003, Pirilä et *al.* 2003). Ln-332 γ2-chain was predominantly present in inflamed CP and G-AgP GCF as 40 kDa and 70 kDa fragments. Furthermore, *P. gingivalis* trypsin-like proteinase (Sorsa et *al.* 1987) produced multiple-size fragments of lower molecular weight and short peptides from mature and unprocessed 100 kDa and 140 kDa Ln-332 γ2-chain species, thus differing clearly from human MMPs that cleave Ln-332 γ2-chain into 40 kDa and 70 kDa fragments. These results suggest that during the procession of periodontitis, bacterial infection resulting in the upregulation and activation of host-derived enzymes, such as MMPs, leads to cleavage of the Ln-332 molecule and the conversion of the junctional epithelium to a diseased pocket epithelium. Study I also demonstrated that the total amount of Ln-332 γ2-chain in the GCF was highest in the CP group. The total amount of Ln-332 also correlated positively with CAL and BOP, and slightly with PD also. These clinical parameters are associated with CP, which is believed to be strongly associated with gingival inflammation (Dale 2002). These results suggest that the host response to microbial infection in periodontal tissues may lead to the altered production of human MMPs and that the human, rather than bacterial proteinases, are predominantly responsible for cleavage of the Ln-332 molecule and for pathological changes in the junctional epithelium.

The Ln-332 concentrations in the GCF were investigated, and the results demonstrated the highest Ln-332 concentration in the healthy group. The presence of Ln-332 in healthy GCF may demonstrate normal physiological basement membrane turnover. GCF volume was significantly higher in both CP and G-AgP groups. Because the total Ln-332 levels correlated positively with GCF volume, and because GCF flow and volume increase during inflammation (Ozkavaf et *al.* 2000), these results confirm that increased GCF volume together with increased levels of Ln-332 eventually reflect the severity and degree of periodontal disease, especially in CP.

In study V, the expression pattern of Ln-332 in a mouse model was evenly distributed in the basement membrane zone of the gingival epithelium. MMP-8 has been demonstrated to cleave Ln-332 at a different cleavage site than do other MMPs, and cleavage with MMP-8 induces no significant or enhanced epithelial cell migration (Pirilä et *al.* 2003). The *WT + P. gingivalis* group exhibited the strongest expression of Ln-332, whereas in the *MMP8*$^{-/-}$ + *P. gingivalis* group the expression was only slightly lower. This result indicates that the role of MMP-8 in Ln-332 processing is not crucial during inflammation, and that other MMPs eventually play a major role in Ln-332 progression.

6.2. MMP-8 levels and activation in GCF and PISF

GCF can be used in the diagnostics and investigation of the severity and degree of inflammation (Ozmeric 2004, Sorsa et *al.* 2004a, 2006). The enhanced expression and activation of various MMP enzymes has been demonstrated in periodontitis as well as in peri-implantitis, where MMPs are believed to mediate multiple functions associated with periodontal destruction and inflammation (Alfant et *al.* 2008, Kivelä-Rajamäki et *al.* 2003). Study III confirmed that MMP-8 activity was, together with GCF and PISF flow, enhanced with increased clinical severity and degree of inflammation in CP and PI. Furthermore, MMP-8 active forms in GCF and PISF contribute to GCF/PISF collagenase acitivity. GCF has been used to evaluate the risk for an individual to develop periodontal disease as well as to monitor the host response to periodontal therapy. MMP-8 levels and activation reportedly to be increase during periodontal inflammation, and MMP-8 can serve as a diagnostic tool to monitor periodontitis (Lamster 1997, Buchmann et *al.* 2002, Kiili et *al.* 2002, Mäntylä et *al.* 2003, 2006, Sorsa et *al.* 2004a, b, 2006). In detecting intra-amniotic infection/inflammation, MMP-8 levels in amniotic fluid have proved to be a useful diagnostic tool (Kim et *al.* 2007, Sorsa et *al.* 2004a, b, 2006, Mäntylä et *al.* 2003, 2006). The present result suggests that PISF could also serve as a diagnostic tool in the same manner as GCF. Furthermore, MMP-8 levels and PISF flow in diseased PI sites were even higher than their respective levels and flow in severe CP sites. Because periodontal and peri-implant inflammation are associated with the extravasation and migration of neutrophils towards the periodontal pocket and an increase in the amount of GCF and PISF volume, these phenomena (increase in PISF flow and MMP-8 activity) could be attributed to even more rapidly progressing inflammation, increased vascular permeability, PMN activity, and influx into the peri-implant mucosal tissues in PI than in CP. These results make PISF interesting and important in the future investigation of the diagnostics of peri-implant inflammations. Study III also demonstrated that the elevation and activation of multiple species of PMN- and fibroblast-type MMP-8 reflect periodontal and peri-implant inflammation, and that MMP-8 is more likely to be produced in many cellular sources rather than in single cellular sources (Hanemaaijer et *al.* 1997, Kiili et *al.* 2002, Tervahartiala et *al.* 2000) in diseased periodontium or peri-implant mucosa.

6.3. MMP-25 and -26 levels and activation in GCF and immunolocalisation in periodontal and peri-implant tissues

MMP-25 is one of the most important MMPs produced by neutrophils and is especially expressed by PMN cells (Pei 1999). Study IV demonstrated that MMP-25 is produced mainly in human plasma cells. In human gingival/mucosal tissue samples, MMP-25 was also found predominantly in plasma cells in CP, AgP, and PI sections, as well as in PMN cell in PI sections. The present findings, together with those of previous studies, confirm that MMP-25 plays a central role in neutrophil function (Pei 1999, Kang et *al.* 2001, Matsuda et *al.* 2003). All MMPs produced/released by neutrophils are important for the invasion and migration of cells to inflammatory sites as well as for the destruction of the host tissue (Pei 1999). MMP-25 is believed to cleave ECM components from the BM, which facilitates the

transendothelial migration of neutrophils during the inflammatory response (Pei 1999, Kang et *al.* 2001). The activation of neutrophils by cytokines or chemokines, such as IL-1β, in the inflammatory response leads to the release of MMP-25 into the extracellular milieu. In studies II and IV, MMP-25 exhibited positive correlation in diseased GCF from CP and G-AgP sites as well as in diseased PI PISF. MMP-25 levels and activation were both enhanced in the GCF and PISF according to the severity of periodontitis/peri-implantitis, which further confirms that in periodontitis- or peri-implantitis-affected oral fluids, MMP-25 is strongly associated with the degree of inflammation.

MMP-26 levels and activation were both enhanced in the GCF/PISF according to the severity of periodontal/peri-implant inflammation. Keratinocytes express MMP-26 proteins in association with various benign skin disorders, in wound repair, and in early skin carcinogenesis (Ahokas et *al.* 2005, Pirilä et *al.* 2007). Therefore MMP-26 may be an inflammatory mediator in some disorders. In study II, CGF MMP-26 immunoreactivity positively correlated with all clinical periodontal parameters. This result confirms for the first time that in the oral cavity MMP-26 is associated with periodontal/peri-implant inflammation and demonstrated more pro- and active forms of MMP-26 in diseased GCF and PISF than in healthy GCF/PISF. Previous studies suggest that MMP-26 can promote the invasion of cancer cells through its activation of pro-MMP-9 (Zhao et *al.* 2003, Uria et *al.* 2000). Elevated levels of MMP-26 in inflamed periodontal/peri-implant tissues and in inflammatory cells suggest that MMP-26 can function as a proinflammatory proteinase in periodontal and peri-implant inflammations as well. GCF MMP-25 and -26 immunoreactivities strongly correlated positively, which further confirms that MMP-26 expression in inflammation is associated with the upregulation of other MMPs, thus promoting the work of these MMP enzymes in concert and cascades at the inflammation site. MMP-26 expression has been demonstrated to be upregulated together with MMP-1 and -7 in odontogenic keratocysts (Cavalcante et *al.* 2008). In human tissues, MMP-26 was strongly present in the BMZ of inflammation-associated CP and PI samples as well as AgP samples, though to a lesser extent. Even though MMP-26 is strongly expressed in normal epithelial and cancer cells of epithelial origin (Marchenko et *al.* 2002, Marchenko et *al.* 2004), MMP-26 has been detected in peripheral leukocytes and lymphocytes (Park et *al.* 2000, Nuttall et *al.* 2003) as well as in fibroblasts and macrophages (Marchenko et *al.* 2004). MMP-26 is known to be associated with cutaneous and mucosal inflammation (Bister et *al.* 2007). The epithelial origin of MMP-26 in the oral cavity during periodontal/peri-implant inflammation together with our results confirm that MMP-26 plays a role in ECM degradation and in the progression of periodontal diseases.

6.4. Immunolocalization of hBDs in inflamed periodontal and peri-implant tissues (IV)

Cutaneous infection or inflammatory diseases enhance hBD production (Niyonsaba et *al.* 2007). In study IV, the inflamed CP and PI tissue sections demonstrated hBD-1 production in oral and sulcular epithelial cells, in endothelial cells, and in the perivascular BMZ. The production of hBD-1 was also found in AgP sections, although to a lesser extent. hBD-1 expression is constitutive in normal physiological conditions, but increases during inflammation (Hata et *al.* 2008). For the first time,

enhanced hBD-1 production in human inflamed periodontal and peri-implant tissues in the oral cavity was demonstrated, which confirms that hBD-1 production is enhanced during inflammation. hBD-1 was also produced in AgP and healthy tissues, though to a lesser extent. Pantelis et *al.* (2008) has suggested that hBD-1 may be a potential tumor suppressor. These results together suggest that hBD-1, in addition to its role in maintaining tissue homeostasis in normal physiological conditions, is also an essential part of the innate immune system in oral infections and inflammatory diseases as well as in non-infectious lesions.

In contrast to hBD-1, no hBD-2 could be detected in the healthy tissues and was only weakly detected in the CP, PI, and AgP tissue samples. hBD-2 can reportedly potentiate skin inflammation by inducing chemotaxis and cytokine and chemokine production (Niyonsaba et *al.* 2007, Yang et *al.* 1999), and several other cytokines are reported to enhance hBD-2 production in keratinocytes (Kanda et *al.* 2008). In addition to antimicrobial properties, hBD-2 can be induced by bacterial and viral products and several cytokines, such as IL-1β, IL-6, IL-8 and TNF-α (Froy 2005). hBD-2 production is enhanced in the proinflammatory response in mucosa (Langhorst et *al.* 2009). Wilson et *al.* (2009) demonstrated that MMP-7 can cleave human neutrophil defensins from propeptides into intermediates, but could not cleave human β-defensins to active forms. This result, however, indicates that MMPs, together with other host enzymes, may play a role in the initiation and activation of host-derived antimicrobial molecules. The results of these recent studies, together with those of the present study (IV), suggest that hBD-2 production is linked to the inflammation and activation of the host response.

hBD-2 reportedly promotes wound healing in the human intestine (Otte et *al.* 2008). In study IV, hBD-2 was produced in the same manner as hBD-1 in the BMZ, as well as in endothelial cells. Baroni et *al.* (2008) recently reported that hBD-2 stimulates the chemotaxis of human endothelial cells to the same extent to that exerted by the vascular endothelial growth factor. hBD-2 also promotes endothelial cell proliferation. In this way, hBD-2 may able to induce wound healing and link inflammation and host defense. Periodontopathogenic bacteria release enzymes and toxins (Bao et *al.* 2008) which may weaken the host response by inactivating and destroying host-derived molecules. Study IV demonstrated for the first time that hBD-1 and -2 were degraded by *P. gingivalis* trypsin-like protease. These results suggest that potent periodontopathogens, such as *P. gingivalis*, can process/modulate or inactivate key molecular mediators or regulators of oral mucosal innate immunity.

6.5. Local and systemic responses to *P.gingivalis*-induced periodontitis in the MMP-8 null-allele mouse model (V)

Previous studies have reported that MMP-8 plays a defensive role in lung inflammation and cancer development (Balbin et *al.* 2003, Gueders et *al.* 2005). MMP-8 deficiency promoted allergen-induced airway inflammation in mice and was associated with elevated levels of several cytokines, such as IL-4, increased neutrophilic and eosinophilic infiltration, and decreased inflammatory cell apoptosis in the lungs (Gueders et *al.* 2005). Owen et *al.* (2004) also demonstrated that active MMP-8 expressed on the

surface of activated PMN regulates lung inflammation in the mice. MMP-8-deficient mice demonstrated greater PMN accumulation in the alveolar space than did WT mice, thus leading to more severe inflammation. The absence of MMP-8 was associated with a marked reduction in the clinical symptoms of experimental autoimmune encephalomyelitis and in central nervous system-infiltrating cells and demyelinating lesions (Folgueras et *al.* 2008). Oral inoculation with *P. gingivalis* induced experimental periodontitis in both MMP-8-deficient and WT mice (V). The *MMP8*$^{-/-}$ mice demonstrated more severe alveolar bone loss than did the WT mice. These *in vivo* results in mice suggest an unexpected anti-inflammatory role for MMP-8 during acute and chronic inflammation. MMP-8 deficiency has recently been demonstrated to increase inflammation and delay wound healing (Gutierrez-Fernandes et *al.* 2007), to play a protective role in tongue cancer development, and to act as a metastasis suppressor by the modulation of tumor cell adhesion and invasion in mice (Gutierrez-Fernandes et *al.* 2008, Korpi et *al.* 2008). Results from mutant MMP-8-deficient mice suggest that total MMP-8 deficiency leads to unexpected changes in ECM metabolism in physiological and pathological infectious and non-infectious conditions. These results suggest that MMP-8 is essential for physiologically healthy tissue, especially collagen turnover, and acts as a regulator for cancer development and inflammation. MMP-8 levels in GCF correlated positively with the severity of periodontal disease (IV). Several other studies have demonstrated that MMP-8 levels in both GCF and PISF correlate positively with periodontitis and peri-implantitis (Kinane et *al.* 2003, Kivelä-Rajamäki et *al.* 2003, Mäntylä et *al.* 2003, Sorsa et *al.* 2006, Rai et *al.* 2008). Golub et *al.* (2008) reported that, compared to the controls, subantimicrobial-dose doxycycline (SDD) reduced 60% of total MMP-8 in GCF. They also reported that SDD therapy significantly reduced collagenase activity, periodontal collagen breakdown, and alveolar bone loss. Administration of the MMP-8 selective inhibitor in mice with experimental autoimmune encephalomyelitis, possibly decreasing the MMP-8 levels back to physiological levels, reduced the severity of the disease (Folgueras et *al.* 2008). Overall, these results suggest that MMP-8 at physiological levels serves protective and anti-inflammatory functions by processing growth factors and protective endogenous proteinase inhibitors (Van Lint & Libert 2006), but pathologically excessive levels are associated with disease progression (Sorsa et *al.* 2004a, 2006). Reducing collagenase levels and activity from pathologically elevated to physiological levels is the next goal in periodontal therapy (Sorsa & Golub 2005).

Both MMP-8 and MMP-9 are reportedly elevated during increases in gingival inflammation (Atilla et *al.* 2001, Rai et *al.* 2008). During inflammation, MMP-8 plays a regulatory role in neutrophil migration, and bacterial LPS can induce both MMP-8 and MMP-9 production (Lin et *al.* 2008). A significant delay in mouse wound closure was observed in *MMP8*$^{-/-}$ mice together with enhanced MMP-9 expression (Gutierrez-Fernandez et *al.* 2007). Furthermore, MMP-8 and -9 form specific complexes *in vivo*. MMP-9 levels were significantly elevated in *MMP8*$^{-/-}$ mice with periodontitis, which further confirms a compensatory MMP-9 upregulation in a situation where MMP-8 is absent. These reports suggest that both MMP-8 and -9 may act co-ordinately during the wound healing process as well as during periodontal inflammation. NE and TNF-α production increased in both infected mice groups. Elastase is involved in the initial degradation of the periodontal ligament in peridontitis (Ujiie et *al.* 2007).

Gingivitis and periodontitis are related to high levels of TNF-α in patients with rheumatoid arthritis (Nilsson & Kopp 2008). The association of NE and TNF-α in *P. gingivalis*-induced periodontitis lesions suggests that the infiltration of PMN cells, with either the increased accumulation or the increased release of lysosomal enzymes, may play a role in the pathogenesis of periodontitis (Figueredo et *al.* 2005). Certain MMPs have been demonstrated to be expressed together with chemokines and chemokine receptors: MMP-2, -9, and -14 expressions led to the consequent activation of TNF-β type I, II, and III receptor complexes (Stover et *al.* 2007), in cancer invasion and metastasis MMP-2-and -9 expressions associated with the chemokine receptor CXCR4 (Zhang et *al.* 2008), and in melanoma metastasis MMP-14 stimulated CXCR4 leading to the activation cascade (Bartolome et *al.* 2009). Furthermore, in *A. actinomycetemcomitans*-induced experimental periodontitis, MMP-1, -2, and -9 are suggested to be involved in TNF-α receptor p55 activation (Garlet et *al.* 2006). Overall, these results suggest that certain MMPs may trigger chemokine receptors leading to increased chemokine expression and activation in inflammation and in cancer.

Studies have demonstrated *P. intermedia* to be related to coronary artery disease (Nonnenmacher et *al.* 2007). Serum total LPS and IgG-class antibody concentrations against *P. gingivalis* were elevated in both infected mice groups, which suggests that systemic exposure of the host to the pathogen and corresponding host responses also accompanied oral infection with *P. gingivalis*. This result further confirms that periodontitis should be considered a risk factor for cardiovascular diseases (Humphrey et *al.* 2008) because periodontitis causes systemic inflammation, and periodontal infection may serve as an inflammatory stimulus that contributes to cardiovascular disease. Oral pathogens may increase the release of cytokines and proinflammatory mediators into circulation that may lead to endothelial damage and cholesterol plaque formation. Bacterial LPS sluff from chronically inflamed periodontal lesions and enter the circulatory system directly, thus affecting the vessel walls.

Decreased HDL cholesterol concentrations have been found in patients with periodontitis (Shimazaki et *al.* 2007). Serum total cholesterol levels decreased during *P. gingivalis* infection; MMP-8 deficiency also reduced apoA-I levels in both infected and uninfected mice. ApoA-I contributes to the reverse cholesterol transport process by interacting with ATP-binding cassette transporter A 1 (ABCA1) in macrophage foam cells and facilitates the efflux of cholesterol (Jessup et *al.* 2006, Tall 2007). Nascent apoA-I HDL is secreted into circulation via ABCA1 (Brunham et *al.* 2006). Because certain MMPs are involved in ABCA1 modification (Tiwari et *al.* 2007), MMP-8 deficiency may also lead to the altered modification of ABCA1, which leads to the disturbed and attenuated secretion of HDL and a reduction in serum. Furthermore, MMP-8 deficiency led to a shift of the HDL population towards a smaller particle size in uninfected *MMP8*$^{-/-}$ mice. These smaller, poorly lipidated HDL particles are more rapidly catabolised from the circulation. These phenomena together may explain the reduced HDL concentrations among *MMP8*$^{-/-}$ mice.

P. gingivalis infection leads to the formation of larger-size HDL and VLDL particles among *MMP8*$^{-/-}$ mice, which is an obvious sign of lipoprotein responses during systemic inflammation. These changes

in serum lipoproteins levels and the increase in HDL/VLDL particle size in *MMP8*$^{-/-}$ mice during *P. gingivalis* infection makes MMP-8-deficient mice more vulnerable to inflammation.

7. CONCLUSIONS

The accelerated expression and activation of Ln-332 and proteolytic enzymes (MMP-8, -25, and -26) was associated with the degree of periodontal and peri-implant inflammation. The expression of antimicrobial hBDs is continuous but tended to be elevated during inflammation. The elevated levels of these molecules and enzymes eventually reflect the extent of the periodontal and peri-implant inflammations, thus suggesting that the host derived molecules can participate in the progression of periodontal and peri-implant inflammatory diseases. These biomarkers may proove to be diagnostically useful tools and also targets of medication in the future. Unexpectably, the presence of MMP-8 causes at least a partially defensive local inflammatory response against the *P. gingivalis* induced development of periodontal bone destruction.

Ln-332, MMP-8, -25, and -26 in GCF and PISF were examined as indicators of the inflammatory host response in different forms of periodontitis and in peri-implantitis. These finding support the conjuncture that GCF and PISF enzymes and molecules are able to distinguish between different forms of periodontal disease and peri-implant inflammation and potentially act as biomarkers of different forms of periodontal and peri-implant diseases in human oral fluids. hBD-1 and -2 expression in human gingival tissues, especially hBD-2 expression, increased during the severity of gingival/mucosal inflammation, suggesting that mucosal antimicrobial peptides play a role in mucosal defense both in killing pathogenic bacteria as well as in inducing cytokine and chemokine production, endothelial cell proliferation, and in co-ordination with other host enzymes, including MMPs in innate immunity. Bacterial proteinases rather than human proteinases can degrade hBDs, thus weakening innate immunity in periodontal and peri-implant infections.

Total MMP-8 deficiency in *P. gingivalis*-induced experimental periodontitis *in vivo* led to enhanced alveolar bone loss and alterations in serum lipoprotein profiles toward more atherogenic profile compared to a situation where MMP-8 is present. MMP-8 eventually participates in periodontitis by contributing to the resolution of inflammation. A reduction from pathologically excessive MMP-8 to nearly physiological levels would be a more desirable goal than the complete inhibition of MMP-8 in the treatment of periodontitis.

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