

Variability of Gingival Crevicular Fluid Matrix Metalloproteinase -8 Levels in Respect to Point-of- Care Diagnostics in Periodontal Diseases

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Lecture Hall 2, Biomedicum Helsinki 1, Haartmaninkatu 8, Helsinki, on 3rd of June 2016, at 12 noon.

Helsinki 2016

ISBN 978-951-51-1996-4 (paperback)

ISBN 978-951-51-1997-1 (PDF)

ISSN 2342-3161 (Print)

ISSN 2342-317X (Online)

<http://ethesis.helsinki.fi>

Unigrafia

Helsinki 2016

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CONTENTS

CONTENTS.....	4
LIST OF ORIGINAL PUBLICATIONS	7
LIST OF ABBREVIATIONS.....	8
ABSTRACT	10
1. REVIEW OF THE LITERATURE	12
1.1. Classification of periodontal diseases	12
1.2. Etiology of periodontal diseases	14
1.3. Pathogenesis of periodontal diseases	16
1.3.1. The role of microbiota in periodontal diseases	16
1.3.2. Host response in the pathogenesis of periodontal diseases.....	17
1.3.3. Innate immunity and neutrophil responses in periodontal diseases ..	18
1.3.4. Adaptive immunity in the pathogenesis of periodontal diseases.....	20
1.3.5. The link between inflammation and bone resorption	21
1.3.6. The modifying effect of smoking on the host response	22
1.4. Matrix metalloproteinases.....	25
1.4.1. Classification and general aspects of MMPs.....	25
1.4.2. Matrix metalloproteinase-8 isoforms.....	26
1.4.3. Regulation of MMP-8 expression and release	27
1.4.4. Activators of latent MMP-8.....	28
1.4.5. Biological functions of MMP-8.....	29
1.4.6. MMP-8 in periodontal diseases.....	30
1.4.7. Genetic polymorphisms related to MMP-8 in periodontal diseases....	30
1.5. Models of the disease progression and the effect of periodontal treatments	32
1.5.1. Natural history of periodontal disease progression - classical studies	
with untreated patients	32
1.5.2. Transitions between healthy, gingivitis and initial periodontitis states	
- statistical Markovian model.....	33
1.5.3. Hierarchy of periodontal data	34
1.5.4. Conventional scaling and root planing treatments.....	34
1.5.5. Host response modulating medical treatments.....	35
1.5.6. Azithromycin as an adjunctive antimicrobial treatment	36
1.6. Oral fluids in periodontal diagnostics	37
1.6.1. General aspects.....	37
1.6.2. Gingival crevicular fluid	38

1.6.3.	Oral fluid MMP-8 in periodontal diagnostics.....	39
2.	AIMS AND HYPOTHESIS OF THE STUDY	52
3.	MATERIAL AND METHODS.....	53
3.1.	Patients and periodontal sites.....	53
3.2.	Clinical periodontal examinations and treatments.....	58
3.3.	GCF Sampling.....	58
3.4.	Biomarker detection methods	59
3.4.1.	MMP-8 time-resolved immunofluorometric assay (IFMA).....	59
3.4.2.	POC MMP-8 detection methods: dentoAnalyzer® and stick-test.....	59
3.4.3.	MMP-8 ELISA.....	60
3.4.4.	MMP-8 western blot.....	60
3.4.5.	Determination of MMP-13, MMP-14, TIMP-1, MPO, Azurocidin, and CXCL5 Levels from GCF.....	61
3.5.	Statistical analysis.....	61
3.5.1.	Statistical comparisons of study groups.....	61
3.5.2.	Data normalization of the pooled data.....	62
3.5.3.	Generalized mixed regression model	64
3.5.4.	Receiver operating characteristics (ROC) analysis	64
3.5.5.	Cluster analysis	64
3.5.6.	Analysis of prognostic utility	65
4.	RESULTS	66
4.1.	Cross-sectional variability in MMP-8 levels.....	66
4.1.1.	Differences between detection methods and comparison of POC methods.....	66
4.1.2.	Differences between healthy, gingivitis and periodontitis sites in MMP-8 isoforms.....	67
4.1.3.	Cross sectional differences between healthy, gingivitis and periodontitis sites in GCF MMP-8 levels.....	67
4.1.4.	Correlations between MMP-8 and the other GCF biomarkers.....	68
4.1.5.	The patient effect on site level GCF MMP-8 levels.....	68
4.1.6.	The effect of smoking on GCF MMP-8 variability.....	68
4.2.	Longitudinal GCF MMP-8 response patterns.....	70
4.3.	Clinical treatment outcomes in longitudinal studies.....	71
4.4.	Diagnostic and prognostic utility of GCF MMP-8 levels	74
4.4.1.	Diagnostic utility and screening performance of GCF MMP-8 and the other studied biomarkers	74
4.4.2.	Prognostic utility of GCF MMP-8 levels.....	75

5. DISCUSSION.....	77
5.1. Random variability and statistical properties of GCF MMP-8 levels.....	77
5.2. Smoking and other modifying effects on GCF MMP-8 levels.....	78
5.3. Comparison of MMP-8 detection methods.....	80
5.4. Determination of clinical treatment outcome with the cluster analysis	81
5.5. Practical implications of GCF MMP-8 test.....	82
6. CONCLUSIONS	88
7. ACKNOWLEDGEMENTS.....	89
REFERENCES	91

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original publications, referred to in the text by their Roman numerals I-IV

- I Sorsa T, Hernández M, Leppilahti J, Munjal S, Netuschil L, Mäntylä P. Detection of gingival crevicular fluid MMP-8 levels with different laboratory and chair-side methods. *Oral Diseases* 2010 January; 16: 39- 45.
- II Leppilahti JM, Hernández-Ríos PA, Gamonal JA, Tervahartiala T, Brignardello-Petersen R, Mäntylä P, Sorsa T, Hernández M. Matrix metalloproteinases and myeloperoxidase in gingival crevicular fluid provide site-specific diagnostic value for chronic periodontitis. *Journal of Clinical Periodontology* 2014 April; 41: 348-56.
- III Leppilahti JM, Kallio MA, Tervahartiala T, Sorsa T, Mäntylä P. Gingival crevicular fluid matrix metalloproteinase-8 levels predict treatment outcome among smokers with chronic periodontitis. *Journal of Periodontology* 2014 February; 85: 250- 60.
- IV Leppilahti JM, Sorsa T, Kallio MA, Tervahartiala T, Emingil G, Han B, Mäntylä P. The utility of gingival crevicular fluid MMP-8 response patterns in prediction of site level clinical treatment outcome. *Journal of Periodontology* 2015 June; 86:777-87.

In addition, this thesis contains some unpublished data.

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Study I: John Wiley & Sons publications

Study II: John Wiley & Sons publications

Study III: The American Academy of Periodontology

Study IV: The American Academy of Periodontology

LIST OF ABBREVIATIONS

<i>A. actinomycetemcomitans</i>	<i>Aggregatibacter actinomycetemcomitans</i>
APC	Antigen presenting cell
AUC	Area under the curve
BOP	Bleeding on probing
CAL	Clinical attachment level
CC	CC chemokine
CD4+	Cluster of differentiation 4 positive
CXCL5	CXC motif chemokine -5
CXCL6	CXC motif chemokine -6
DTPA	Diethylenetriaminepentaacetic
ECM	Extracellular matrix
ENA-78	Epithelial-derived neutrophil-activating peptide 78
fMLP	Formyl-methionyl-leucyl-phenylalanine
GCF	Gingival crevicular fluid
GI	Gingival index
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule -1
ICTP	C-telopeptide pyridinoline cross-links
IFN- γ	Interferon gamma
IL	Interleukin
kDa	kilo Dalton
LDD	Low dose doxycycline
LIX	LPS induced CXC chemokine
LPS	Lipopolysaccharide
LR	Likelihood ratio
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NET	Neutrophil extracellular trap
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
OPG	Osteoprotegerin
PAMP	Pathogen associated molecular patterns

PD	Pocket depth
<i>P. gingivalis</i>	<i>Porphyromonas gingivalis</i>
<i>P. intermedia</i>	<i>Prevotella intermedia</i>
POC	Point-of-care
PRR	Pattern recognizing receptors
PSD	Polymicrobial synergy and dysbiosis
RANK	Receptor activator of NF- κ B
RANKL	Receptor activator of NF- κ B ligand
ROS	Reactive oxygen species
RSF	Rheumatoid synovial fibroblasts
SRP	Scaling and root planing
<i>T. denticola</i>	<i>Treponema denticola</i>
<i>T. forsythia</i>	<i>Tannerella forsythia</i>
Th	T helper cell
TIMP	Tissue inhibitor of matrix metalloproteinase
TLR	Toll like receptors
TNF- α	Tumor necrosis factor alpha
Treg	Regulatory T cell
Tris	Tris(hydroxymethyl)aminomethane
WB	Western blot

ABSTRACT

Matrix metalloproteinases (MMP) and especially MMP-8 is one of the most widely reported oral fluid biomarkers and a promising target candidate for periodontal point-of-care (POC) diagnostics. Periodontitis is associated with increased oral fluid MMP-8 levels, which typically decrease after conventional periodontal treatments. Employing the measurement of oral fluid MMP-8 levels diagnostically, however, is complicated due to high variability. Chronic periodontal inflammation can induce MMP-8 expression in a wide array of cell types, although a great extent of the MMP-8 detected from the gingival crevicular fluid (GCF) originates from polynuclear neutrophil granulocytes (neutrophils). MMP-8 mediates periodontal tissue breakdown by processing extracellular matrix (ECM) proteins with a wide substrate specificity. There is also increasing evidence that MMP-8 contributes to inflammatory signaling cascades and has a protective role in periodontitis and cancer. Thus, it is important to define and differentiate a (statistical) range of physiological fluctuations in the oral fluid MMP-8 POC diagnostics, from the pathologically high MMP-8 levels.

The aim of this study was to study methodological and biological reasons for the large variability of GCF MMP-8 and further to evaluate the utility of GCF MMP-8 for POC diagnostics and its ability to predict the treatment outcome after the conventional scaling and root planing (SRP) treatment and during the supportive maintenance period. Different laboratory and POC MMP-8 detecting methods were compared to study methodological reasons of variability in the GCF MMP-8 levels. In addition, correlations between different inflammatory GCF biomarkers and MMP-8 were compared.

The MMP-8 levels measured with laboratory methods or POC tests/devices based on the same monoclonal antibody were clearly in agreement and correlated significantly. There was surprisingly large variability in MMP-8 levels, however, when measured with different assays based on different antibodies.

The comparison of different GCF biomarkers revealed highly discriminating properties, especially for myeloperoxidase (MPO) and MMP-8, to differentiate both healthy and gingivitis sites from moderate to advanced chronic periodontitis sites.

The longitudinal variability in GCF MMP-8 response patterns was explored and the prognostic utility of GCF MMP-8 was studied. Distinct response patterns during the maintenance period could be found via cluster analysis, especially among smoking patients. High MMP-8 levels at baseline and especially the high-responding pattern among smokers during the maintenance period predicted the compromised treatment outcome.

The utility of the GCF MMP-8 levels in the prognostic POC diagnostics was further studied within a heterogenic population by combining different independent datasets. Continuously high MMP-8 levels, at baseline and during the maintenance period, predicted an increased risk for compromised treatment outcome for both non- smoking and smoking patients. Low MMP-8 levels decreased the risk respectively.

Overall, the different MMP-8 antibodies can have a quite large difference in affinities to different MMP-8 isoforms, causing variability to the measured levels. If researchers fail to use the same detection methods in different studies, result comparisons may be complicated. GCF MMP-8, however, is a promising candidate as a prognostic biomarker to identify sites with an increased risk for compromised treatment outcome. This study also strongly supports the evidence that MMP-8 can diagnostically differentiate between periodontitis and healthy or gingivitis sites and can also be used for the quantitative, therapeutic monitoring of treatment outcome. Different GCF MMP-8 cutoff levels should be applied for smokers and non-smokers in the MMP-8 based POC diagnostics, however.

1. REVIEW OF THE LITERATURE

1.1. Classification of periodontal diseases

Periodontal diseases have been classified as either non-destructive (gingival and reversible) or destructive diseases and further etiologically into different sub categories. For non-destructive, “gingival”, diseases, the principle subcategories are “plaque-induced” and “primarily non-plaque-induced” lesions. Plaque-induced diseases are further classified by different modifying factors, which define over 40 different gingival diseases and conditions in the classification system. (Armitage1999)

Periodontitis, in general, refers to the bacteria-induced, inflammatory, destructive periodontal diseases associated with the breakdown of the tissues surrounding teeth. The pathogenesis of periodontitis comprises different etiological risk and modifying factors. According to a classification system published in 1999, destructive periodontal diseases are classified into seven principal categories. These categories include: i) chronic periodontitis, ii) aggressive periodontitis, iii) periodontitis as a manifestation of systemic disease, iv) necrotizing periodontal disease, v) abscesses of the periodontium, vi) periodontitis associated with endodontic lesions, and vii) developmental or acquired deformities and conditions. This thesis study focuses on the first two categories: chronic periodontitis and aggressive periodontitis. Chronic periodontitis is the most common form of destructive periodontal disease.

The 1999 classification system has been criticized for being impractical due to complex and multiple main and sub categories. Comparisons between different independent studies proves difficult when the extent and severity of periodontitis cases lack precise definitions (van der Velden2005). Another major clinical challenge is to distinguish between destructive periodontal diseases, especially aggressive and chronic periodontitis, because the exact moment of disease onset is often unknown. Aggressive periodontitis usually affects younger patients but there is no definitive age relatedness in the disease. According to the epidemiological knowledge and the classification system based on it, chronic periodontitis cannot rule out aggressive periodontitis in the course of disease, i.e. chronic periodontitis can turn into aggressive periodontitis. On the other hand, the “chronic” attribute does not imply that it is untreatable (Armitage1999). Much research has been pursued to define inflammatory or microbial biomarkers that could

differentiate between chronic and aggressive periodontitis, however no single biomarker or composite of markers is yet known to differentiate between the disease types (Armitage2013).

Van der Velden (2005) argued for a nominalistic classification system in contrast to an essentialistic system aiming to categorize a disease according etiological factors. In the nominalistic system, a certain disease label is given based on well-defined signs and symptoms of the disease. The nominalistic classification could be more practical in the case of periodontitis with the common signs and symptoms of the diseases but with the complex and variable etiological factors. Van der Velden (2005) suggested a nominalistic classification system based on four dimensions: extent (number of affected teeth), severity (amount of attachment/bone loss per tooth), age (if the moment of disease onset is known) and clinical characteristics (necrotizing, rapidly progressive, or refractory periodontitis for example). The nominalistic classification would facilitate better population comparison between epidemiological studies. The fifth European consensus workshop in periodontology has also given a proposal, based on the extent and the severity of disease, for the periodontitis case definition to be implied in epidemiological studies (Tonetti et al. 2005).

Smoking- and diabetes-associated periodontal diseases have been discussed regarding whether they should be classified as independent entities (Lopez & Baelum2012). However, the classification system has not been collectively renewed since the 1999.

1.2. Etiology of periodontal diseases

Periodontal diseases are considered etiologically complex. An important notion in an epidemiological causal theory and model (sufficient-component cause model) for complex and multi causal diseases is that a single risk factor cannot cause the disease in isolation (Heaton & Dietrich2012). Causal etiological risk factors of destructive periodontal diseases, referred to here as periodontitis, can be divided into modifiable and non-modifiable factors. Modifiable risk factors include: environmental, acquired, and behavioral; whilst non-modifiable risk factors include: age, gender and heredity (genetic and epigenetic factors) (Borrell & Papapanou2005).

Three modifiable factors, including high-level colonization by specific bacteria (*A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*), smoking, and uncontrolled diabetes, are considered to be established risk factors for chronic periodontitis based on consistent evidence from multiple independent epidemiological studies (Borrel & Papapanou2005). In addition, there are many other putative modifiable factors, such as socio-economic factors, HIV, obesity, osteopenia/osteoporosis, vitamin D deficiency, and psychosocial factors, supported by a variable amount of evidence (Page & Kornman1997, Borrell & Papapanou2005, Stabholz et al. 2010, Genco & Borgnakke2013). The modifying effects of smoking in relation to the pathogenesis of periodontal diseases and immunity are addressed in more detail in a later chapter.

There is a specific list of systemic diseases with the manifestation of periodontitis classified as an own disease category (Armitage 1999). The same main category also includes the sub category of periodontitis associated with genetic disorders (familial neutropenia, Papillon- Lefèvre syndrome, down syndrome etc.), which is not addressed here when referred to the genetic susceptibility of periodontitis.

It is well documented that the prevalence of periodontitis varies in different ethnic groups and geographic locations. Especially aggressive periodontitis is more common in the African population and in descendants of Africans (1-5%) compared to Caucasians (0.1-0.5 %), Asians (0.2-1%), or Hispanics (in North America, 0.5- 1%) (Susin et al. 2014). However, the difference in prevalence of periodontitis between different ethnic groups is not so consistent if age, behavioral and socio economic factors are taken into account (Borrel & Papapanou2005). There is also much evidence from different populations that

periodontitis is more frequent in males compared to females. There is insufficient evidence to declare male gender as a risk factor for the onset of disease, but men have a higher risk for severe forms of the disease. The difference is explained with behavioral aspects. Plausible biological mechanisms, an effect of sex hormones on the immune system for example, that may explain the difference, are yet to be described, but cannot be ruled out. (Borrell & Papapanou 2005, Haytac et al. 2013).

The evidence gathered from twin and familial aggregation studies suggest that genetic factors can explain a major part of variance in population prevalence of both chronic and aggressive periodontitis (Michalowicz et al. 1991, Michalowicz et al. 2000, Laine et al. 2012). However, there have been arguments that the genetic influence on disease severity may have been overestimated in earlier twin studies (Torres de Heens et al. 2010).

A few candidate genes have been found from case-control studies associating single nucleotide polymorphisms (SNPs) in genes with chronic (IL-1 β , IL-1RN, IL-6, IL-10, CD14, vitamin D receptor, MMP-1 and TLR4) or aggressive periodontitis (IL-1 β , IL-1RN, Fc γ RIIb, vitamin D receptor, TLR4) reviewed by Laine et al. (2012). However, an increasing number of candidate genes, varying in different populations/studies, are being explored in the era of genome-wide association studies (GWAS) (Schaefer et al. 2010, Divaris et al. 2013, Teumer et al. 2013, Schaefer et al. 2014, Kallio et al. 2014).

1.3. Pathogenesis of periodontal diseases

1.3.1. The role of microbiota in periodontal diseases

Periodontitis, in general, is considered to be a polymicrobially-induced inflammatory disease with a destructive host response against the putative periodontal pathogens. There is an abundant literature and a long history of studies that associate certain bacteria (*P.gingivalis*, *T.denticola*, *T.forsythia*, *A.actinomycetemcomitans* etc.), and an ecological shift (dysbiosis) of biofilm structure, with periodontitis (Haffajee & Socransky1994, Socransky et al. 1998, Socransky & Haffajee2005). Nevertheless, the development of culture-independent detection methods and high-throughput sequencing have revealed a large bacterial diversity and enhanced our understanding of the role of bacteria in periodontitis (Kumar et al. 2005, Colombo et al. 2009, Griffen et al. 2012). The theory of “red complex” putative periodontal pathogens, according to the classical study of Socransky et al. (1998), has evolved to a model of polymicrobial synergy and dysbiosis (PSD-model) (Hajishengallis & Lamont2012). The need to update the paradigm has arisen from the fact that the putative periodontal pathogens also frequently associate with healthy periodontium. The PSD-model does not deny the importance of the “red complex” or the other putative pathogens in periodontitis but it focuses on their role and better describes the mechanism in disruption of balance between host responses and microbes in periodontal diseases (for example, *P. gingivalis* as a keystone pathogen) (Darveau et al. 1998, Hajishengallis et al. 2011, Hajishengallis et al. 2012). It has also been discussed whether the association between the putative periodontal pathogens and the dysbiosis, an increased proportion of gram- negative anaerobes, and periodontitis is a cause or result of inflammation (Bartold & Van Dyke2013, Hajishengallis & Lamont2012). The dysbiosis and the success of putative periodontal pathogens in periodontitis sites could also be a result of chronic inflammation, which can change the environment in the periodontal sulcus to favor the growth of asaccharolytic bacterial species. An increased flow of GCF from inflamed periodontal tissue carries amino acids and peptides, breakdown products from inflamed tissue, which are nutrients especially for many putative periodontal pathogens, the asaccharolytic gram-negative anaerobes (Bartold & Van Dyke2013).

1.3.2. Host response in the pathogenesis of periodontal diseases

Microbes are obligatory in the induction of (plaque induced) periodontal diseases, but equal importance should be placed on the alteration of the immunological host response against the microbiota in the development and progression of different types of periodontitis. There is a continuous interplay between the periodontal microbiota and defense mechanisms and immune system around the gingival crevice (Hajishengallis2014). According to the epidemiological model, the host-response-related component causes can be considered equally important in the initiation and progression of periodontitis. The term component cause is synonymous with the terminology of risk factor (Heaton & Dietrich2013). Putative periodontal pathogens are considered to be necessary, but not solely sufficient, cause to initiate periodontitis. The host-response-related component causes can vary from patient to patient and form, together with the causal pathogens, classes of sufficient causes, a constellation of periodontal diseases associated with partly different component causes, i.e. risk factors.

Many of the host-response-related, established (uncontrolled diabetes and smoking) and putative components causes (e.g. obesity/metabolic syndrome and vitamin D deficiency), can cause or at least are associated with changes/modifications in the immune system. Smoking as a risk factor and modifying effect on host response is described below in more detail as a major object of this thesis. Immunity is schematically divided into the innate and the adaptive immune systems, irrespective of whether prior learning is required to activate the system. A classical histopathological study of the different types of periodontal diseases revealed a gradual shift from the innate-immunity-related cell types in the initial and the early gingivitis lesions to the adaptive-immunity-associated plasma cells and the general features of chronic inflammation in the established gingivitis and the advanced periodontitis lesions (Page & Schroeder1976). However, in a longstanding chronic inflammatory condition, both immune systems can be considered to work together with diverse feedback mechanisms that impact each other (**Figure 1**). Different aspects of the immune system, related to the pathogenesis of periodontal diseases, which may cause biological variability to MMP-8 levels, are reviewed in more detail in the following chapters.

1.3.3. Innate immunity and neutrophil responses in periodontal diseases

The innate immune system operates as an inherent defense mechanism that does not require prior learning. It is permanently active, even in healthy periodontal sites, evident from a continuous flow of neutrophils through the junctional epithelium (Page & Schroeder1976, Scott & Krauss2012).

Innate-immune-system triggered inflammation is activated after periodontal pathogens have invaded through the first line of defense in the periodontium; comprising intact junctional epithelium and a constant flow of GCF and antibacterial products. Pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) link the innate and the adaptive immune systems together by recruiting and activating the adaptive immune system related cells (Preshaw & Taylor2011, Ebersole2003, Kinane et al. 2011). (**Figure1**)

Innate defense systems became established during a long evolutionary relationship between multicellular species and bacteria. This has resulted in an inherent capability of the innate immune system to recognize distinct microbial structures, so called pathogen associated molecular patterns (PAMPs) (Hajishengallis2014, Di Benedetto et al. 2013, Kinane et al. 2007, Preshaw & Taylor2011). These patterns are identified by the pattern recognizing receptors (PRR), such as the toll-like receptors (TLR) that are expressed in diverse cell types (neutrophils, monocyte lineage cell, specialized T cells, dendritic cells, epithelial cells)(Kinane et al. 2007). TLR and other PRR are important in host-microbe interactions to regulate inflammatory responses appropriate for commensal vs. pathogenic periodontal microbes (Kinane 2007). Recognition of virulent pathogens leads to: pro-inflammatory chemokine and cytokine production in sentinel cells (macrophages, dendritic cells, epithelial cells etc.); complement activation and recruitment; the emigration of circulating neutrophils; and further stimulation of the adaptive immune responses (Di Benedetto et al. 2013, Kinane et al. 2007, Hajishengallis2014, Krauss et al. 2010).

Neutrophils are considered to be key players in the protection of the periodontium against the microbiota. Neutrophils form a barrier around the sulcular epithelium, and their balanced functions are important in the maintainance of health in periodontal tissues. Even healthy periodontium has a small but constant flow of neutrophils through the epithelium into the gingival crevice (Page & Schroeder1976) (**Figure 1**). Adverse neutrophil counts and function-related disorders have been associated with periodontitis. Decreased counts of circulating neutrophils (neutropenia), impaired

functions with normal neutrophil counts, and hyper-reactive or constitutively hyperactive neutrophils are all associated with periodontitis (Scott & Krauss2012, Nussbaum & Shapira2011).

During the maturation period in bone marrow, neutrophils synthesize different proteolytic enzymes (i.e. elastase, MMP-8, MMP-9), antimicrobial proteins (i.e. azurocidin, lactoferrin) and reactive oxygen species generating MPO and store them in the different granules (Borregaard et al. 2007). The coordinated release of the granule content causes ECM breakdown, enables neutrophil migration (MMP-8, MMP-9, elastase), and facilitates extracellular antimicrobial actions. Nevertheless, the neutrophil-derived factors also have many inflammation-regulating effects (Borregaard2010, Van Lint & Libert2006, Hajishengallis2014). The infiltrating neutrophils release many pro-inflammatory cytokines, (IL-8, alarmins), and the ECM-degrading proteases can modulate chemokine functions (Borregaard2010, Nauseef & Borregaard2014). The neutrophil-originated proteinases, MMP-8 and MMP-9 for example, can cleave LPS-induced CXC chemokines (LIX), CXC motif chemokine -5 [CXCL5, called also epithelial-derived neutrophil-activating peptide 78 (ENA-78)] and -6 (CXCL6), to more potent N-terminally truncated form (Van Lint & Libert2006, Van Den Steen et al. 2003). In addition, neutrophils secrete the alarmins (i.e. β -defensin, azurocidin), an endogenous equivalent to PAMP, which recruit monocytes and other antigen presenting cells (APC) (Soehnlein & Lindbom2009). Azurocidin is a unique antimicrobial neutrophil-derived protein. It belongs evolutionarily to the serine proteinase super family but has lost its proteolytic capability through mutation. Azurocidin is stored in both the primary granules and the secretory vesicles, which might indicate different functions. In recent studies, azurocidin has also been proven to have signaling, alarmin-like functions in addition to the long-known microbicidal effects (Soehnlein & Lindbom2009).

Neutrophils kill bacteria through either by phagocytic action and producing reactive oxygen species inside the phagosomes, or by extracellular degranulation and secretion of microbicidal products. Neutrophil extracellular trap (NET) is a more recently found antimicrobial function by neutrophils (Brinkmann et al. 2004). NETs are comprised of the extracellular strands of DNA bound with neutrophil-derived proteins. NETs are suggested to be produced by a phenomenon called NETosis, which is regarded as an alternative cell death besides apoptosis and necrosis (Nauseef & Borregaard2014). The microbicidal effects of NETs are controversial but are considered to at least restrain

viable bacteria and limit free diffusion of granule-released proteases and tissue damage (Nussbaum & Shapira2011). Elastase, MPO, and ROS are essential for NET formation (Nauseef & Borregaard2014). Neutrophils of patients with Papillon-Lefèvre syndrome, a rare genetic disorder, cannot support the NETosis because the lack of elastase and other serine proteases. However, number of studies related to NET production in periodontitis is low and the role of NET production remains unclear in relation to chronic periodontitis.

1.3.4. Adaptive immunity in the pathogenesis of periodontal diseases

Periodontitis is histologically described by characteristic plasma-cell infiltration (Page & Schroeder1976, Berglundh & Donati2005). An activation and proliferation of B lymphocytes to plasma cells is a part of the adaptive immunological responses. Adaptive immunity is stimulated by pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α , for example) recruiting circulating lympho- and monocytes followed by antigen processing and presentation by antigen presenting cells (APCs) (Preshaw & Taylor2011, Kinane et al. 2011). Dendritic cells and macrophages have traditionally been described as APCs but B cells also have antigen presenting functions (Berglundh & Donati2005). Dendritic cells are key players between the innate and adaptive immune systems. They digest, process and sample antigens from peripheral tissues and migrate to secondary lymph organs mainly lymph nodes to present antigen samples on the major histocompatibility complex (MHC) II molecules for naïve B and T cells (Ebersole et al. 2013a).

Antigen presentation to naïve CD4+ cells activates T helper (Th) cell differentiation to Th1, Th2, or Th17 effector-cell types or regulatory T cells with distinct cytokine profiles. The cytokine networks related to Th-cell differentiation are summarized in **Figure 1**.

Much research has focused on the roles of cell-mediated (Th1) and humoral (Th2) immunity in periodontitis. Histopathological studies indicated that periodontitis lesions trigger a predominantly Th2 response with plasma-cell-rich infiltration. It has been proposed that Th2 response predispose the host to disease progression as it is inefficient to maintain the innate immunity against periodontal microbiota. It also promote IL-1 β facilitated, osteoclast-mediated, bone resorption. However, an opposite hypothesis has also been proposed. (Page & Schroeder 1976, Gaffen & Hajishengallis 2008, Gemmell et al. 2002)

The Th1 vs. Th2 paradigm of periodontitis has been refined by the roles of Th17 and developmentally close induced regulatory T cells (iTreg) (Hajishengallis2014, Gaffen & Hajishengallis2008). Th17 acts near the epithelial lining to promote epithelial cells to produce antimicrobial products and strengthen neutrophil responses and innate immunity against extracellular microbes (Hajishengallis2014). Th17 acts through IL-17, which indirectly (through CXC chemokines) induce neutrophil production in the bone marrow and chemotactic recruitment into the periodontal tissues (Hajishengallis2014). There is also a positive feedback loop between neutrophil-secreted chemokines (CXCL2 and CXCL20) and the differentiation and the recruitment of Th17 cells into the periodontal tissues (Hajishengallis2014, Preshaw & Taylor2011). Regulatory T cells, in contrast, prevent excessive inflammatory responses by expressing anti-inflammatory cytokines, transforming growth factor (TGF- β), and IL-10, which suppress Th1, Th2, and Th17 function. The role of Th17 response, beside the Th1 and Th2 responses, must be investigated further to understand the role of Th17 in the broad manifestations of periodontal diseases (Preshaw & Taylor2011, Gaffen & Hajishengallis2008).

1.3.5. The link between inflammation and bone resorption

The breakdown of soft tissue structures in periodontitis is considered to be caused by MMPs and other proteases secreted by inflammatory cells: neutrophils, macrophages, T cells, B cells, and plasma cells. Chronic inflammatory conditions also induce fibroblast and epithelial cells to express MMPs (Sorsa et al. 2006, Hanemaaijer et al. 1997). An absolute requirement for bone loss in periodontitis is osteoclast activity and the differentiation of osteoclast precursors to active osteoclasts by the stimulation of receptor activator of NF- κ B (RANK) on the cell surface of osteoclast precursors with RANK ligand (RANKL). An inhibiting counterpart for RANKL is osteoprotegerin (OPG), which can block RANK activation. (Belibasakis & Bostanci2012)

Many cells, such as fibroblasts and osteoblasts, are capable of expressing RANKL, but B and T cells are a major source of soluble RANKL (Belibasakis & Bostanci2012). Some studies have demonstrated that neutrophils can express cell-membrane RANKL under some circumstances but, to facilitate the bone loss *in vivo*, neutrophils should migrate to a close proximity with the bone (Chakravarti et al. 2009).

1.3.6. The modifying effect of smoking on the host response

Smoking is a major risk factor for periodontitis and has diverse systemic effects, which can modify the host response in periodontal diseases (Palmer et al. 2005). Short-term vasoconstrictive effects on skin capillaries are detected in response to nicotine after smoking, although no similar effect has been shown in gingival blood flow or results have been contradictory (Palmer et al. 2005). However, there is probably also a long-term vasoconstrictive effect on gingival blood flow. Morozumi et al. (2004) showed that after a successful smoking cessation, periodontal blood-flow levels increased significantly during the following weeks. Gingival blood flow was measured in these studies with a laser Doppler device, which can be used to detect only relative changes in blood flow but not to compare absolute levels of blood flow between patients. Nevertheless, the relative blood flow changes and the GCF volume changes correlated clearly during the follow-up, and the GCF volumes were comparable between the nonsmokers and the smokers two weeks after quitting smoking (Morozumi et al. 2004). In histological samples, non-smoking periodontitis patients have had a larger number of blood vessels compared to smoking patients (Rezavandi et al. 2002, Palmer et al. 2005). Lower levels of intercellular adhesion molecules (ICAM) -1 and E-selectin positive vessels have also been detected in smokers compared to non-smokers, which might have had an effect on neutrophil transmigration (Rezavandi et al. 2002, Palmer et al. 2005).

The effect of smoking on gingival inflammation and bleeding on probing has been shown in many large epidemiological studies (Borrell et al. 2005, Eke et al. 2012). For instance, in an experimental intervention study, in which a pro-inflammatory effect of plaque accumulation was observed, smokers developed a clinically detectable reduced inflammatory response compared to the non-smokers (Bergström & Preber 1986).

Smoking triggers a wide range of effects on neutrophils, including increased circulating neutrophil counts (Palmer et al. 2005). On the other hand, levels of the neutrophil-derived proteases (elastase, MMP-8) and other products are reduced in GCF, indicating a reduced neutrophil transmigration to the gingival crevice or an impaired function (Mäntylä et al. 2006, Ding et al. 1994, Heikkinen et al. 2010, Ozcaka et al. 2011).

Peripheral neutrophils from smoking subjects have been shown to release an increased amount of free radicals after priming with TNF- α compared to non-smokers (Gustafsson et al. 2000). Smoking alone had no significant effect on released radicals after the stimulation, but periodontitis itself seemed to own a priming effect on neutrophils

(Fredriksson et al. 1999). In more recent studies, cigarette-smoke-extract reduced neutrophils' ability to generate reactive oxidative species (ROS) after the stimulation but at high concentration the extracts caused an increased extracellular release of ROS, and authors concluded a dual effect of smoking on periodontitis: the impaired elimination of bacteria with smaller concentrations of ROS and, in heavy smokers, the oxidative stress caused by the increased ROS release (Matthews et al. 2011, Matthews et al. 2012).

Tobacco smoking can also have a modulatory effect on immune reactions. Decreased counts of infiltrated dendritic cells have been measured among smokers. Nicotine affected dendritic cells and the differentiation of CD4+ T cells to Th1, Th2, or Th17 cells when stimulated with LPS (Yanagita et al. 2012). Naïve CD4+ T cells were more probably differentiated to Th2 cells when exposed to nicotine, as without nicotine, Th1 cells were the more probable outcome (Yanagita et al. 2012, De Heens et al. 2009). These results fit well with the histopathological picture of the dominant plasma-cell presence in periodontitis infiltrate (Page & Schroeder 1976).

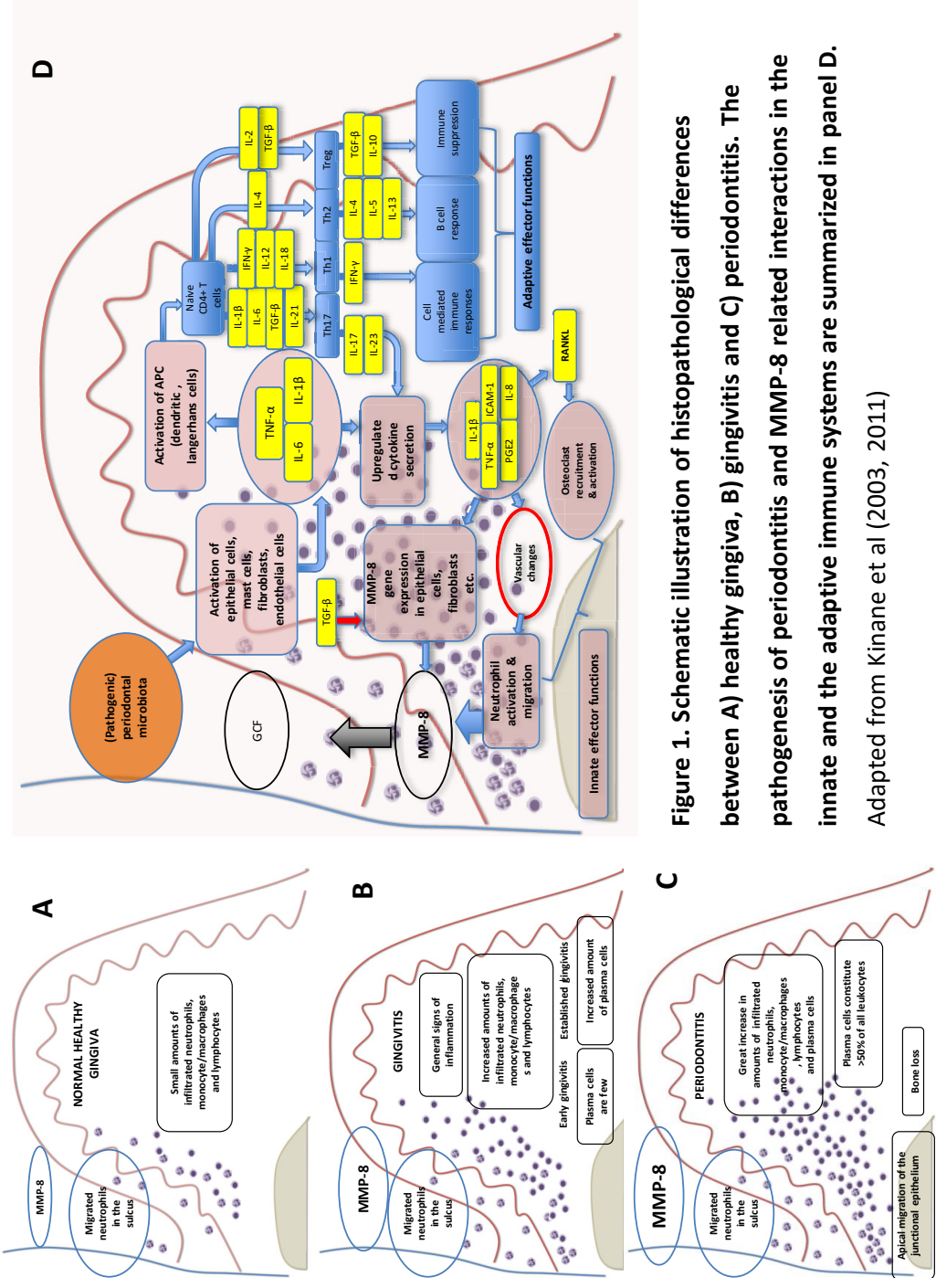


Figure 1. Schematic illustration of histopathological differences between A) healthy gingiva, B) gingivitis and C) periodontitis. The pathogenesis of periodontitis and MMP-8 related interactions in the innate and the adaptive immune systems are summarized in panel D.
 Adapted from Kinane et al (2003, 2011)

1.4. Matrix metalloproteinases

1.4.1. Classification and general aspects of MMPs

The proteolytic actions of MMPs were first described by Gross & Lapiere (1962), who identified the collagenolytic activity in the metamorphosis of amphibian tadpoles (Gross & Lapiere 1962). Today, the MMP family is known to comprise 25 structurally similar but partly genetically distinct proteolytic, zinc-dependent endopeptidases reviewed by Sorsa et al. (2006), Visse & Nagase (2003), and Nagase et al. (2006). Most MMPs are extracellular proteinases secreted in an inactive pro-form but membrane bound MMPs also exist and certain MMPs can also be activated intracellularly. The structure of MMP is composed of similar basic components: the propeptide, the catalytic metalloproteinase domain, and the hemopexin domain. There are some exceptions, however, and divergence exists in the structure and function of different MMPs (Sorsa et al. 2006, Visse & Nagase 2003, Nagase et al. 2006).

MMPs are classified according to their substrate specificity and structure into six groups: *collagenases* (MMP-1, -8, -13); *gelatinases* (MMP-2, -9); *stromelysins* (MMP-3, MMP-10, -11); *matrilysins* (MMP-7, -26); *membrane-type MMPs* (MMP-14, -15, -16, -17, -25); and *other MMPs*: macrophage elastase (MMP-12), MMP-19, enamelysin (MMP-20). Genes coding human collagenases, including MMP-8, are located near to each other on chromosome 11 (11q22-q23). (Sorsa et al. 2006, Nagase et al. 2006, Visse & Nagase 2003).

The activity of MMPs is controlled through multiple levels of regulation from gene expression to compartmentalization, pro-enzyme activation, and the inhibition of activated MMPs. The catalytic domain of MMPs with Zn-ion is blocked with the bond between the ion and the cysteine switch in the pro-peptide of latent MMP. The *in vivo* activation of latent MMPs occurs when the bond between the cysteine switch and Zn-ion is interrupted by either a proteolytic cleavage of the pro-peptide or by chemical modifications or reactions with oxygen species formed abundantly during inflammation. (Sorsa et al. 2006, Nagase et al. 2006, Visse & Nagase 2003)

Activated MMPs are further regulated through inactivation by the non-specific inhibitors (alpha-2-macromolecules) and specific tissue inhibitors (TIMPs) (Sternlicht & Werb 2001). Alpha-2-macroglobulins are large glycoproteins, which inhibit MMPs by

binding their own proteinase component (Werb et al. 1974). Alpha-2-macroglobulins are the main inhibitors of MMPs in liquid phase, plasma for example (Visse & Nagase2003). TIMPs, in contrast, are smaller, more specific inhibitors that bind to the catalytic domain of MMPs (Visse & Nagase2003, Sternlicht & Werb2001). Altogether four different TIMPs are known and each of them has their specific target MMPs. In addition, TIMPs have been shown to have other biological activities (Visse & Nagase2003). TIMP-1 is the most important inhibitor of active MMP-8 in periodontal tissues (Sorsa et al. 2006).

1.4.2. Matrix metalloproteinase-8 isoforms

MMP-8 has been isolated (Sorsa et al. 1985, Sorsa1987) and sequenced (Hasty et al. 1990, Knäuper et al. 1990a), at first from neutrophils, and historically was called neutrophil collagenase. However, several cell types from different tissues have been proved to express MMP-8. MMP-8 protein and gene expression *in vivo* has been shown to occur in synovial and gingival fibroblasts (Hanemaaijer et al. 1997), cartilage chondrocytes (Chubinskaya et al. 1999), epithelial cells (Tervahartiala et al. 2000, Pirilä et al. 2001, Prikk et al. 2001), monocytes/macrophages (Prikk et al. 2001), and plasma cells (Wahlgren et al. 2001, Kiili et al. 2002). *In vitro*, even more MMP-8 expressing cell types have been found (Sorsa et al. 2006).

There are several MMP-8 isoforms with different molecular weights: The latent and the active glycosylated neutrophil MMP-8 with the molecular weight (Mr) of 70-75 kDa and 50-60 kDa. The non-glycosylated, *de novo* secreted, “mesenchymal” forms have the molecular weight of around 50-40 kDa, whilst lower molecular weight fragments also exist (Hanemaaijer et al. 1997, Kiili et al. 2002).

Owen et al. (2004) showed that only 15-20% of cellular MMP-8 was freely released in soluble form after a pro-inflammatory “physiological” stimulation (TNF- α or PAF+fMLP) and after the stimulation a marked amount of MMP-8 was bound to the outer surface of extracellular membrane facilitating neutrophil migration, for example. However, MMP-8 lacks the membrane binding domain, which is specific to the membrane-type (MT) MMPs and the attachment of “membrane bound” MMP-8 had to be achieved with another mechanism. (Owen et al. 2004)

Owen et al. (2004) also further studied differences in MMP-8 isoforms between the cell compartments. Freely released, extracellular, soluble MMP-8 was inevitably in the latent form with a molecular weight of 85kDa. Inside the specific granules, MMP-8 was also detected in the 110, 40, and 30 kDa isoforms, in addition to the major 85kDa form. Plasma membranes contained, in turn, MMP-8 in the latent 85 kDa, the active 65 kDa, and the fragmented 30 kDa isoforms, and also the minor forms of 110 kDa, 80 kDa, and 46 kDa. (Owen et al. 2004)

Differences in the glycosylation of neutrophil-derived MMP-8 may explain the differences in molecular weights between studies. Hanemaaijer et al. (1997) showed the difference in the molecular weights of MMP-8 from purified neutrophils extracts or the rheumatoid synovial fibroblasts (RSF) and the human umbilical vein endothelial cells (HUVEC) culture media in the western blot before and after the deglycosylation. Before the deglycosylation, the neutrophil extracts contained MMP-8 isoforms comparative to the molecular weights of around 75 kDa, also referred above. After the deglycosylation, the neutrophil extracts contained four bands on the western blot of about 70, 65, 50, and 45 kDa in weight and also the minor fragmented forms around 30 kDa. RSF and HUVEC culture media only had the 50 kDa form on the western blot and deglycosylation had no significant effect on the molecular weight of MMP-8. Glycosylation is most probably important for the compartmentalization of MMP-8 into specific neutrophil granules. (Hanemaaijer et al. 1997)

1.4.3. Regulation of MMP-8 expression and release

IL-1 β has been shown to induce MMP-8 expression in gingival fibroblasts (Abe et al. 2001), and TNF- α in endothelial cells (Hanemaaijer et al. 1997), *in vitro*. IL-6 has been shown to increase MMP-8 expression in myeloma cells (Wahlgren et al. 2001) and TGF β -1 has been shown to decrease MMP-8 expression in odontoblasts *in vitro* (Palosaari et al. 2000). TGF β -1 levels have also been more increased in MMP-8 knock- out mice with a wound healing model suggesting feedback mechanisms between TGF β -1 and MMP-8, interestingly (Åström et al. 2014, Balbin et al. 2003). In addition, hyperoxia has been shown to promote up-regulation of MMP-8 expression (Cederqvist et al. 2006).

In neutrophils, MMP-8 gene expression occurs during the maturation period in the bone marrow and the latent protein is stored inside the specific granules (secondary

granules) (Bainton et al. 1971, Borregaard et al. 2007). Neutrophil-originated MMP-8 is highly glycosylated, which is assumed to relate to the compartmentalization of MMP-8 during the neutrophil maturation period (Knäuper et al. 1990b, Hanemaaijer et al. 1997). The degranulation of neutrophils and the amount of MMP-8 released to an extracellular space is affected by the priming of different cytokines (IL-1 β , TNF- α) and the stimulus of bacteria derived products (LPS) or other chemical stimulus (oxidative species) (Sorsa et al. 2006).

1.4.4. Activators of latent MMP-8

The activation of latent MMPs can occur with the proteolytic cleavage as described above. The following MMPs or other proteolytic host enzymes can activate the latent MMP-8 by the proteolytic modification of pro-domain: MMP-10 (stromelysin-2) (Knäuper et al. 1993, Knäuper et al. 1996), MMP-3 (stromelysin 1) (Knäuper et al. 1993), MMP-14 (Holopainen et al. 2003), MMP-7 (Dozier et al. 2006), serine protease cathepsin G (Kähäri & Saarialho-Kere 1999, Tervahartiala et al. 1996) and trypsin -2 (Moilanen et al. 2003).

In addition to proteolytic cleavage, MMP-8 can also be activated by reactive oxygen species (Sorsa et al. 1989, Suomalainen et al. 1991, Saari et al. 1990, Saari et al. 1992, Weiss et al. 1985). The susceptibility of the neutrophil MMP-8 to ROS is believed to be due to the high degree of glycosylation in the neutrophil isoforms (Sorsa et al. 2006). MPO, which catalyzes the production of hypochlorous acid (HOCl) from hydrogen peroxide (H₂O₂) and chloride anion (Cl⁻), is released from the stimulated neutrophils. Hypochlorous acid is a potent MMP-8 activator and the coordinated degranulation of MPO and the other neutrophil proteases is believed to promote the activation of the latent extracellular MMP-8 (Saari et al. 1992, Springman et al. 1990, Sorsa et al. 2006).

In addition to host-derived activating factors, the proteolytic cleavage and the ROS activation of MMP-8, bacterial species have been noted to promote collagenase activity; especially *T. denticola* and *P. gingivalis* derived proteases, extracted from the periodontal sulcular plaque (Sorsa et al. 1992, Sorsa et al. 1995, Ding et al. 1996, Ding et al. 1997).

1.4.5. Biological functions of MMP-8

MMP-8 has a wide substrate specificity overlapping with other collagenases. MMP-8 cleaves collagen types-I, -II -III, -VII, and -X, with the order of specificity to collagen I>III>II (Hasty et al. 1987), and a wide array of other ECM substances (Sorsa et al. 2006, Van Lint & Libert2006). ECM contains a large amount of latent chemokines and bioactive proteins, which are released and activated after proteolytic actions of proteases, including MMPs in inflamed periodontal tissue, and leads to complex pro- and anti-inflammatory cascades of gene expression and inflammatory cell migration. (Van Lint & Libert2006).

MMP-8 has been revealed to contribute to anti-inflammatory defensive effects in experimental models of skin cancer, inflammatory lung diseases, and also in periodontitis (Balbin et al. 2003, Owen et al. 2004, Gueders et al. 2005, Kuula et al. 2009). MMP-8 knockout (KO) mice with *P. gingivalis*-induced periodontitis demonstrated more alveolar bone loss compared to wild-type controls, indicating that MMP-8 has a protective physiological role in inflamed tissues (Kuula et al. 2009). Excessively expressed levels associate with the active and the unstable disease (Lee et al. 1995, Mäntylä et al. 2003, Mäntylä et al. 2006, Sorsa et al. 2015). The anti-inflammatory effects of MMP-8 probably result from the processing of chemokines released from degraded ECM. In the wound-healing model, MMP-8 KO mice exhibited a more diffuse neutrophil migration in the tissues compared to the wild type controls (Gutierrez-Fernandez et al. 2007). In addition, the KO mice were not able to clear out the infiltrated neutrophils causing a prolonged inflammatory response (Gutiérrez-Fernández et al. 2007). Thus, MMP-8 can have an effect on the neutrophil recruitment during the up-regulation of inflammation, but also the resolution of inflammation in the later periods (Van Lint & Libert 2006). The ability of MMP-8 and MMP-9 to cleave LPS induced CXC chemokine (LIX) to a more potent neutrophil attractant is proposed as one possible explanation for the different inflammatory-response patterns in the MMP-8 KO and the wild type mice (Balbin et al. 2003, Van Den Steen et al. 2003, Van Lint & Libert2006, Hernandez et al. 2011). However, no direct human *in vivo* relationship between MMP-8 and LIX have yet been shown (Van Lint & Libert 2006).

1.4.6. MMP-8 in periodontal diseases

Most of the MMP-8 in gingival tissues and GCF is most likely derived from neutrophils, although many other cells can express and secrete MMP-8 after chronic pro-inflammatory stimulus as described above. Studies including gingival samples with immunohistochemical staining of MMP-8, and expressing strong correlations with pure neutrophil-associated GCF biomarkers (elastase, azurocidin, MPO) give support for this view (Sternlicht & Werb 2001, Uitto et al. 2003, Beklen et al. 2007, Hernandez et al. 2010)

A major function of the neutrophil-driven neutral proteases, including MMP-8, is to enhance neutrophil migration in (periodontal) tissues toward an infection focus-driven by the chemo-attracting signal gradients. In MMP-8 knockout mice with *P. gingivalis*-induced periodontitis, neutrophils are distributed diffusely when compared to the wild types, which may indicate less efficient actions against the microbes (Kuula et al. 2009). The degradation of connective tissue enables the inflammatory signaling molecules, chemo- and cytokines etc., to spread out more efficiently (Uitto et al. 2003). Thus, the effect of MMP-8 in periodontal diseases can be described as a double-edged sword. Certain physiological levels of MMP-8 are necessary for efficient neutrophil functions and the regulation of inflammatory response in periodontitis, while excessive levels cause more tissue breakdown and are associated with a more severe disease and disease progression (Lee et al. 1995, Mäntylä et al. 2003, Mäntylä et al. 2006, Kinney et al. 2014, Ramseier et al. 2009).

1.4.7. Genetic polymorphisms related to MMP-8 in periodontal diseases

Three single nucleotide polymorphisms, with possible effects on MMP-8 gene expression, have been identified to date in the promoter region of the MMP-8 gene at the positions -799 C/T (rs1320632), -381 A/G (rs11225395), and +17 C/G (rs2155052) (Ye 2000). The -799 T allele carriers have been associated with aggressive periodontitis in the Taiwanese population (Chou et al. 2011) and generalized aggressive periodontitis (G-AgP) in the Turkish population when compared to healthy controls (Emingil et al. 2014). The haplotype -799 T/ +17C has been also associated with CP (Izakovicova Holla et al. 2012). However, these SNPs have not been identified in genome-wide association studies of chronic or aggressive periodontitis patients (Schaefer et al. 2010, Divaris et al. 2013, Teumer et al. 2013, Schaefer et al. 2014, Kallio et al. 2014).

In the study of Emingil et al. (2014), polymorphisms in both MMP-8 and TIMP-1 genes were compared between G-AgP patients and healthy controls in the Turkish population and the treatment outcomes between the different genotypes were also studied. The conclusion of the study was that the MMP-8 -799 C/T and the TIMP-1 372 T/C, *429 T/G gene polymorphisms could associate with the susceptibility to G-AgP in males. Small differences in the GCF MMP-8 levels were also reported between the genotypes three months after SRP therapy.

1.5. Models of the disease progression and the effect of periodontal treatments

1.5.1. Natural history of periodontal disease progression - classical studies with untreated patients

Patterns of periodontitis disease progression have been studied for decades. Classical epidemiological longitudinal follow-up studies have given an impression of a linear continuous process of attachment loss, in which the progression rate can vary in different populations and by modifying risk factors (Löe et al. 1978). The annual average rate of attachment loss in the linear model was approximated to be around 0.1mm with the different-aged, randomly-recruited Norwegian males regularly treated by a dentist and 0.2 – 0.3 mm in the Sri Lankan population with no dental treatments or preventive interventions (Löe et al. 1978). In the cohort studies, like the above described, intervals between the follow-ups are usually relatively long, several years, and fluctuations of individual or site-specific disease activity between the surveys cannot be detected. (Socransky et al. 1984a, Schätzle et al. 2009, Mdala et al. 2014)

In another classical longitudinal study by Goodson et al. (1982), untreated periodontitis patients were followed once a month during one year. The majority of sites (83%) displayed no significant change in the probing measures during the follow-up and most of the sites with significant change (increase or decrease of probing measures) became shallower, when the patterns of disease progression and regression were analyzed (Goodson et al. 1982). In another study, untreated patients were followed bimonthly during one year. Five percent of sites were observed to lose attachment and gain was shown in 2% of sites (Haffajee et al. 1983). Even smaller percentage rates were observed if a more conservative statistical method was used to define the significant clinical attachment level (CAL) change within the data (Haffajee et al. 1983). A challenge faced in the analysis of longitudinal data is that the detected significant attachment loss or the pocket deepening is not permanent during the follow-up (Goodson et al. 1982, Haffajee et al. 1983). From the sites with significant pocket depth (PD) increase, approximately half retained the deep levels during the follow-up as the rest of sites had spontaneous remission (Goodson et al. 1982). Further on, the same authors noticed that in a longitudinal study of six years, the annual attachment loss was on average 0.18 mm,

comparable with rates in the linear model (Löe et al. 1978), and only 12% expressed the attachment loss of more than 2 mm during the period (Socransky et al. 1984). On the other hand, 40% of sites that exhibited the attachment loss during the first 3 year period, had no change in the next 3 years and 50% of sites with no change in the first 3 years, had attachment loss in the last 3 years (Socransky et al. 1984). Comparable results were shown in a more recent cohort study of 12 years (with 6 years examination intervals) from New Zealand. Only 13.2% of sites were observed to have moderate or markedly increasing attachment loss during the 12 years follow-up (Thomson et al. 2013). Socransky et al. (1984) proposed, based on the above described discrepancy between the estimated annual rates of the linear disease progression and the observations of disease progression and regression in relatively short intervals between the examinations, that the progression of periodontitis is more a burst-like phenomenon than a continuous process.

1.5.2. Transitions between healthy, gingivitis and initial periodontitis states – statistical Markovian model

The cross sectional mean levels and the changes in respective means of attachment loss fail to definitively describe the patient and site specific fluctuations between gingivitis and periodontitis lesions during the follow-up. Recently, a more sophisticated statistical analysis of transitions between healthy or gingivitis status to periodontitis and reverse, i.e. both progression and regression, have been conducted (Schätzle et al. 2009, Mdala et al. 2014). Schätzle et al. (2009) used the multistate Markov model to estimate probabilities of transitions from healthy to gingivitis and initial periodontitis and reverse during the 7 visits of a cohort followed up for 26 years (the same cohort as in Löe et al. 1978 was followed). Authors concluded that increased mean calculus index and smoking were significant predictive covariates for the progression, while increased mean gingival index (GI) and younger age predicted the regression of initial periodontitis (Schätzle et al. 2009). Authors explained the regressive effect of high GI, a bit paradoxical finding compared to earlier results from the same authors (Schätzle et al. 2003), with smoking. Smoking probably suppressed inflammatory response and GI, and the smokers were more susceptible to the accumulation of plaque and calculus (Schätzle et al. 2009).

Mdala et al. (2014) used a similar model to describe transitions of healthy sites to gingivitis and periodontitis in a two-year-long clinical trial, which was originally designed

to study the effect of different periodontal treatments and adjunctive antimicrobial medications on chronic periodontitis patients. Because the follow-up was only 2 years, reverse transitions from periodontitis to gingivitis or healthy sites were not allowed in the model. Healthy sites retained their state for an average 32 months; 87-97% of transitions were from healthy to gingivitis as a smaller proportion was classified as the fast transition from healthy to periodontitis. Smoking and disease severity were significant risk factors for the fast transitions (Mdala et al. 2014).

1.5.3. Hierarchy of periodontal data

Another major challenge in statistical analyses of periodontal data is the complex structure including many levels of hierarchy: site, tooth, and patient level, and repeated measures from same sites in longitudinal studies. In this regard, the site level measures (including site-specific biomarkers) are not truly independent in statistical analysis. The hierarchy can be taken into account with multi-level models or mixed models (Gilthorpe et al. 2003, Tu et al. 2004). Gilthorpe et al. (2003) employed such a model to analyze CAL changes in 100 patients and 4 sites of all teeth in a longitudinal study of three years, with three examination visits. They concluded that the changes in CAL levels were cyclical during the follow-up but changes exhibited the dynamic regression to the mean at both the tooth and the patient level. This means that both the linear and the burst models of disease progression are a manifestation of the same phenomenon depending on the perspective and time scale.

1.5.4. Conventional scaling and root planing treatments

The conventional treatment by scaling and root planing (SRP), and supportive maintenance treatment, reduce microbial load, absolute quantity, and relative proportion of the putative periodontal pathogens, and should further lead to resolution of inflammation. However, it is a well-known fact that not all sites and patients, although a minority, respond well to a given treatment and treatment results cannot be maintained after the initial treatments (Drisko2001). Insufficient instrumentation, poor oral hygiene compliance and neglect of maintenance visits have been considered major factors for the compromised treatment outcome. However, the constitutional and environmental factors affecting host response may also be reasons for recurrent periodontitis and the

non-responding and the prolonged periodontal inflammation after the treatments (Drisko2001).

Meta-analysis of SRP treatment has shown PD reduction on average to be around 1 mm in sites with moderate periodontitis lesion (4-6mm) and 2 mm in advanced lesions (>6 mm) as mean CAL gain values has been 0.5 mm and 1 mm, respectively (Hung & Douglass2002). The greatest change in PD and CAL values occurs from one to three months and the evaluation of treatment outcome is not recommended before one month after the treatment. Healing and improvement in clinical parameters can continue even several months after the initial treatment (Cobb2002).

1.5.5. Host response modulating medical treatments

There is much evidence promoting the role of host response in the pathogenesis of periodontitis and also the host-response-modifying treatment options have been developed. The effect of low-dose doxycycline (20 mg twice a day) is based on the chelation property of doxycycline toward Ca^{2+} and Zn^{2+} dependent proteases (MMPs) (Golub et al. 1985, Golub et al. 1990, Ingman et al. 1993, Suomalainen et al. 1992, Ashley1999). Doxycycline also suppresses MMP gene expression (Ramamurthy et al. 1999). In fact, the association of decreased MMP-8 levels with long (several months) LDD or antimicrobial tetracycline treatments is probably related to the suppressed gene expression more than blocking MMPs; blocking does not necessarily have an effect on the affinity of an MMP-8 specific antibody to detect MMP-8 levels (Emingil et al. 2004, Golub et al. 2008, Reinhardt et al. 2010, Payne et al. 2011, Kormi et al. 2014).

The clinical effect of LDD has been found in multiple studies and the United States Food and Drug Administration has approved LDD as an adjunctive medication with SRP for periodontal treatment (Caton & Ryan2011). Significant differences have been reported in mean PD reduction and CAL gain in patients with SRP adjunctive with LDD compared to placebo (Caton & Ryan2011). Clinically, the proportion of sites with significant (>2 mm) PD reduction and CAL gain is higher in LDD treated patients compared to placebo (Caton & Ryan2011, Preshaw et al. 2005, Preshaw2008). In some studies, non-smokers seemed to benefit more from LDD medication compared to smokers (Preshaw et al. 2005, Needleman et al. 2007).

1.5.6. Azithromycin as an adjunctive antimicrobial treatment

Azithromycin is a wide spectrum antibiotic against aerobic and anaerobic gram-negative putative periodontal pathogens (Herrera et al. 2008, Muniz et al. 2013). There has been an increasing interest in azithromycin usage as an adjunctive medication, together with SRP in periodontal diseases because azithromycin has some beneficial pharmacological properties (Foulds et al. 1990, Muniz et al. 2013). One 500 mg tablet of azithromycin taken once a day for three days is a dosage used to achieve sufficient therapeutic levels in tissues for 7 days (Gomi et al. 2007). On the other hand, a long half-life of azithromycin may be a risk for the development of resistant species, at least shortly after taking the medication (Haffajee et al. 2008). Azithromycin has also been shown to carry anti-inflammatory properties in addition to its anti-microbial effect (Culic et al. 2001). However, somewhat contradictory results have been reported in relation to the effect of adjunctive use of azithromycin compared to placebo. Muniz et al. (2013) have reviewed clinical double- or single-blinded trials in relation to the adjunctive use of azithromycin and they concluded that most studies reported significantly reduced PD and increased attachment gain compared to controls. However negative results with no significant differences between the placebo and azithromycin have also been reported (Dastoor et al. 2007, Han et al. 2012a, Emingil et al. 2012, Sampaio et al. 2011). Some studies suggest that azithromycin might have a beneficial effect in the treatment of advanced sites with >6 mm deep pockets when the instrumentation is not necessarily sufficient to remove all microbial deposits (Emingil et al. 2012).

1.6. Oral fluids in periodontal diagnostics

1.6.1. General aspects

Oral fluid based diagnostics is drawing increasing scientific interest in periodontal research but also in research related to systemic diseases, such as cardiovascular diseases or cancer (Sorsa et al. 2004, Sorsa et al. 2006, Sorsa et al. 2011, Sorsa et al. 2015). Oral fluids have certain diagnostic advantages, compared to blood samples. Most importantly, oral fluid sampling is non-invasive and a simpler procedure to carry out. Oral fluid samples comprise saliva, oral rinse, or gingival crevicular fluid samples. Saliva sampling can be further divided to stimulated and non-stimulated saliva samples. All of the methods have certain advantages and disadvantages from the perspective of periodontal point-of-care diagnostics. Saliva sampling is probably the easiest to perform and there is no requirement for dental-professional aid if simple instructions are followed. The oral rinse is near to the saliva sampling (Pauletto et al. 2000, Romanelli et al. 1999, Mancini et al. 1999). The main difference is that, in the oral rinse sampling, saliva is washed out from the oral cavity by rinsing the mouth with water vigorously before the actual sampling. The actual oral rinse sample is collected after the washout by rinsing the mouth again with a standard amount of water for a standard time period (Romanelli et al. 1999, Mancini et al. 1999). This way, the content of GCF is highlighted in the mouth-rinse sampling compared to the saliva sample, which is more influenced by the salivary gland secretions. GCF is a site-specific sampling method and is less biased by any other possible oral pathological conditions (Uitto et al. 2003). Actually, GCF is considered to be completely distinct exudate or transudate of gingiva and is not mixed with saliva until GCF is flown out of the crevice (Griffiths2003). A disadvantage of GCF sampling is that it is technically more demanding compared than saliva or oral-rinse sampling and dental professionals are required to perform the sampling to avoid contaminations (Griffiths2003). Also, the amount of obtained GCF in mild gingivitis and healthy sites is quite small and a detection of some biomarkers from low levels of GCF can be difficult.

Based on the work developed by Wilson et al. (2007), Kinane et al. (2011) grouped biomarkers into five groups according to information the groups give about periodontal diseases and if there is any evidence for biomarkers to belong to these groups.

- 1) *Susceptibility*: “A biomarker that prospectively identifies individuals or sites at increased risk for periodontal disease”.
- 2) *Diagnostic*: “A biomarker that identifies the presence of periodontal disease”.
- 3) *Prognostic*: “A biomarker that identifies patients or sites most likely to respond to specific interventions”.
- 4) *Predictive*: “A biomarker that predicts future progression of disease.”
- 5) *Therapeutic*: “A biomarker that provides a quantifiable measure of response to periodontal therapy”

1.6.2. Gingival crevicular fluid

GCF is an exudate or transudate filtrated from periodontal tissues into the periodontal sulcus (Griffiths2003). The amount of GCF production is affected by capillary permeability, lymphatic drainage of interstitial fluid and an osmotic pressure in the sulcus formed by bacterial products, host-derived proteins, and other molecules. In healthy tissues, capillary permeability is small, most of the interstitial fluid is drained into the lymphatic vessels and only a small amount of GCF is transuded into the sulcus. In healthy tissues, the osmotic pressure affects the formation of GCF relatively more, compared to inflamed tissues where the capillary permeability is increased and the lymphatic draining cannot take care of the increased interstitial hydrostatic pressure, and the excess of the interstitial exudate is filtrated into the sulcus. There is a difference in protein contents between exuded and transuded GCF. The Exuded GCF from inflamed periodontium has large co-variation with serum, as the transuded GCF form healthy tissues remind more interstitial fluid. (Griffiths2003)

GCF resting volumes can be ten-fold higher in moderate pockets (0.4 μ l, 4-5 mm) and even a 30-fold increase has been observed in deep pockets (1.5 μ l, 6-9 mm) compared to healthy sites (0.05 μ l). GCF flow rates have been reported to increase from 3 μ l/h in healthy sites to 44 μ l/h in deep pockets. (Goodson2003)

Significantly lower GCF volumes have been detected among smokers on average, but after smoking ceased, GCF volume recovered to levels comparable with non-smokers

within two weeks (Morozumi et al. 2004). GCF volume correlated with increasing gingival blood flow during the non-smoking period of smokers measured with a laser doppler flow meter (Morozumi et al. 2004).

1.6.3. Oral fluid MMP-8 in periodontal diagnostics

Many studies have sought to detect periodontitis-associated biomarkers from GCF. MMP-8 is one of the most studied and reported periodontal biomarkers. A PubMed search with MESH keywords "*periodontal disease*" and "*GCF*" produced in total 1090 articles (published before 2015). A combination of "*MMP-8*" ("*MMP-8*" or "*collagenase - 2*" or "*neutrophil collagenase*") with the before mentioned words produced 90 articles. Respective searches for IL-1 β was 195, for elastase 86, for TNF- α 74, for MMP-9 36, and MMP-13 produced 16 results.

In addition to the search ("*periodontal disease*" and "*GCF*" and "*MMP-8*"), the keywords "*periodontal disease and MMP-8* (or collagenase or neutrophil collagenase)" produced 226 studies. There were a few GCF MMP-8 articles, which could be found with the "*periodontal disease and MMP-8*" search but not with the "*periodontal disease and GCF and MMP-8*" search.

After a manual check of both searches, 103 MMP-8 and GCF and human periodontitis related articles were found and further categorized into four groups of studies: i) clinical trials, ii) cross sectional (periodontitis) case-control studies and correlation studies of clinical periodontal measures and GCF MMP-8 levels, iii) longitudinal studies comparing progressive or active sites vs. stable, and iv) experimental studies related to the MMP-8 biological functions (the activation of latent pro-enzyme, the direct biological actions, correlations of GCF MMP-8 with other biomarkers, the effect of modifying factors on GCF biomarkers etc.)

Longitudinal studies/clinical trials and cross sectional case-control and correlation studies were reviewed in more detail. All included clinical trials (n = 44), the effect of SRP was studied alone or in conjunction with host response modifying medication (LDD) or some other adjunctive treatment (laser, surgery, NSAID, antibiotics, etc.). The vast majority of studies reported decreased average MMP-8 levels, with or without the adjunctive treatment. In 72% of articles, MMP-8 levels were reported to decrease significantly after SRP treatment (alone) during variable follow-up periods. In the

reviewed studies, with the combination of the SRP and LDD, a significant GCF MMP-8 reduction was reported in 89% of studies and in 76% of studies if SRP was combined with the other medication or treatment (no LDD), respectively. (**Table 1**)

The above described search of 103 GCF MMP-8 original articles included 33 cross-sectional case-control or correlation studies. All the cross sectional studies are presented in **Table 2**. Studies were grouped into three sub-categories according to how clinical parameters or disease status were compared with GCF MMP-8: i) A correlation between GCF MMP-8 levels and clinical parameters was analyzed, ii) MMP-8 levels between healthy and periodontitis sites or patients (pooled GCF) were compared, or iii) GCF MMP-8 levels between gingivitis and periodontitis sites/patients were compared. Almost all studies found a statistically significant association/correlation between at least some of the studied clinical parameters (PD, CAL, and BOP for example). Altogether 86% of studies comparing MMP-8 levels between healthy and periodontitis sites (or pooled GCF from healthy and periodontitis patients) reported significantly increased MMP-8 levels in periodontitis sites or patients. On the other hand, only 56% of studies reported significantly higher GCF MMP-8 levels in periodontitis sites/patients compared to gingivitis.

There are relatively few studies comparing GCF MMP-8 levels in progressive or the active sites to the stable periodontitis sites in the longitudinal cohorts. The vast majority of clinical trials listed in **Table 1** were designed to study the treatment effect and not the disease progression or the detection of active sites. The studies designed to study the association between the active sites or the disease progression and GCF MMP-8 are listed in **Table 3**. One study out of five could not find no significant difference in GCF MMP-8 levels between active/progressive and stable sites as the rest of studies reported increased MMP-8 levels in active/progressive periodontitis sites (**Table 3**).

The vast majority of the studies analyzed differences between periodontal disease groups and controls with statistical tests and reported p-values. Few studies reported the diagnostic performance of GCF MMP-8, also with sensitivity/ specificity, odds ratios, or likelihood ratios (**Table 1** and **Table 2**) (Mäntylä et al. 2003, Kim et al. 2014).

Table 1. Effect of periodontal treatment on GCF MMP-8 levels in clinical trials

Author	Year	N of patients x (sites)	The Effect of SRP treatment + possible adjunctive treatment			MMP-8 detection method	Additional information
			alone	LDD	Other		
Suomalainen	1992		(+)			Collagenase activity	
Golub et al.	1997	18 x (8-12)	-	+		Collagenase activity	
Said et al.	1999	32 x (2-3)			(-)	IFMA	Surgery + biomembr + amoxicillin 2g
Ashley et al.	1999	75 (171)	-	+		Collagenase activity	
Sorsa et al.	1999	(31)	+			IFMA	
Chen et al.	2000	16 x (7-16)	+			IFMA	
Okuda et al.	2001	36			+	ELISA (Amersham)	Surgery + emdogain
Azmaq et al.	2002	20 x (2)	-		+	IFMA	SRP + CHX chip
Budunelli et al.	2002	22	+		+	IFMA	SRP + meloxicam
Persson et al.	2003	30 x (3)			(+)	ELISA	Surgery

Kinane et al.	2003	20 x (4)	+		IFMA
Lee et al.	2004	41 x 4	-		ELISA (Amersham)
Figueredo et al.	2004	64 x (5-6)	+		ELISA (Quantikine)
Emingil et al.	2004	20	+	+	IFMA
Mäntylä et al.	2004	19	(+)		IFMA
Choi et al.	2004	32	(-)	+	ELISA (Amersham)
Qadri et al.	2005	34		(-)	SRP+ laser
Pozo et al.	2005	13 (60)	(+)		Collagenase activity + WB
Mäntylä et al.	2006	16 (132)	+		IFMA
Ağan et al.	2006	10 (32)	+	+	ELISA (Quantikine)
Kurtis et al.	2007	58	+	+	SRP + CHX SRP+ NSAID
Qadri et al.	2007	40		-	SRP + Laser
Correa et al.	2008	46 x (5-6)	(+)		ELISA (R&D) MMP-8 reduction only in deep pockets
Golub et al.	2008	64 x2	(+)		Westernblot
Hernandéz et al.	2010	50	+		IFMA

Qadri et al.	2010	60	(+)	(+)	ELISA (Systems Europe)	SRP+ laser
Tuter et al.	2010	58 (348)	+	+	ELISA	
Marcaccini et al.	2010	42	+		ELISA	
Basegmez et al.	2011	160	+	+	ELISA	SRP + Minocyclin
Han et al.	2012	28	+	+	IFMA	SRP+ Azithromycin
Gilowski et al.	2012	34	-	+	ELISA (Quantikine)	
Eltas & Orbak	2012	40	+	+	ELISA (Amersham)	SRP + Laser
Konopka et al.	2012	51	+		ELISA (Amersham)	
Emingil et al.	2012	32	+	+	IFMA	SRP+ Azithromycin
Gonçalves et al.	2013	29 (58)		+	Fluorometric assay	SRP+ antibiotic
Farhad et al.	2013	39 (117)	-	+	ELISA (R&D)	
Queiroz et al.	2013	20 (40)	-	-	MMP-8 (Quantikine)	SRP + Laser
Alves et al.	2013	62 x 4	+		Bio-Plex assay	
Özgören et al.	2014	32 x 4	-	+	ELISA	SRP+NSAID
Kinney et al.	2014	100	+		Cytokine Array Raybiotech	

Saglam et al.	2014	30	+	+	Raybiotech	SRP + Laser
Pourabbas et al.	2014	22 x 2	-	-	ELISA	SRP + Laser
Tsalikis et al.	2014	66	+	+	ELISA (Quantikine)	

Significant GCF MMP-8 decrease

(+) after treatment **26 (72%)** **8 (89%)** **16 (76%)**

No significant change in GCF MMP-8 levels

(-) after treatment **10 (26%)** **1 (11%)** **5 (24%)**

The effect of treatment on GCF MMP-8 levels is indicated with +/- signs. The + sign indicates significant MMP-8 decrease after the treatment. The - sign indicates no significant change in the MMP-8 levels, respectively. (+/-) sign within the brackets indicates controversy in results

Table 2. Cross-sectional case-control association studies and correlation studies of GCF MMP-8 and clinical parameters or disease severity

Study	Year	N of patients (x sites)	Correlation with clinical parameters	Significant difference between**		MMP-8 detection method	Additional information
				H vs. P	G vs. P		
Lee et al.	1995	58	+		+	Collagenase activity	
Halinen et al.	1996	9		(-)		Collagenase act. & WB	
Ingman et al.	1996	34		+		ELISA	
Nomura et al.	1998	21	(+)	(+)		Collagenase act. & WB	
Söder	1999	40 x 4	+				
Mancini et al.	1999	163	(+)	(+)	(+)	Biotinylated-collagen assay	
Romanelli et al.	1999	19+25	(+)	(+)	(+)	Biotinylated-collagen assay	
Chen et al.	2000	16	+			IFMA	
Atilla et al.	2001	39x2	(+)			IFMA	Cyclosporin med.

Kiili et al.	2002	12	+		WB	
Mäntylä et al.	2003	29 (207)	+	+	IFMA	
Ullbro et al.	2004	28	(+)	(+)	ELISA	Pap. Lefevre vs. control
Pozo et al.	2005	24 (84)	+		Collagenase activity & WB	
Figueredo et al.	2005	110		(-)	ELISA (Quantikine)	Gingivitis vs shallow pockets
Söder et al.	2006	64x4	+		ELISA (Amersham)	
Passoja et al.	2008	64x2	(+)		ELISA (Quantikine)	Shallow pockets
Prescher et al.	2007	64	(+)	(+)	DentoAnalyzer & IFMA	
Alfant et al.	2008	84 (144)	+		Collagenolytic activity	
Bildt et al.	2008	20	+		MMP activity/ zymography	
Emingil et al.	2008	143 (336)	+		IFMA	Tacrolimus med
Teles et al.	2009	40 (931)	-	-	Checkerboard	
Biyikoğlu et al.	2009	74 (148)	+	-	IFMA	

Marcaccini et al.	2010	42	+	+	ELISA
Teles et al.	2010	40 (931)	+	-	Checkerboard
Kardeşler	2010	73 (146)	+	-	IFMA
Rai et al.	2010	20 x (1-4)	+	+	ELISA
Rai et al.	2012	107 (408)	+	+	ELISA (Quantikine)
Kraft-Neumärker et al.	2012	9 (92-112)	+	+	IFMA
Konopka et al.	2012	51	+	+	ELISA
Han et al.	2012	314	+	+	ELISA
Yakob et al.	2013	99 (396)	(+)	-	IFMA Assoc. with <i>T.Denticola</i>
Alves et al.	2013	62 (248)	+	+	Bio-Plex assay kit
Kim et al.	2014	506	+	+	ELISA (R&D)
Significant correlation/ association (+)			16	18 (86%)	5 (56%)
No correlation/ association (-)			0	3 (14%)	4 (44%)

A significant association/correlation between the MMP-8 levels and studied clinical parameters is indicated with + sign and no association with - signs, respectively. A sign within brackets indicates a controversy in results. Healthy (H), gingivitis (G), periodontitis (P).

P-values fail to clarify diagnostic utility because they are affected by both, the differences between the group and the sample size in the study. The sensitivity and specificity are probably the most common statistical measures used to measure diagnostic performance. The measures of sensitivity and specificity are affected by a chosen cutoff. The ROC analysis provides a relationship between true (sensitivity) and false positives (1-specificity) with different cutoffs. Odd ratios, likelihood ratios, and positive and negative predictive values are other possible measures that provide information about the diagnostic utility of GCF biomarkers.

One additional PubMed search (“*periodontal disease*” and “*MMP-8*” and “*sensitivity and specificity*”) was performed to find articles with diagnostic performance calculations, also for the utility of saliva MMP-8 in periodontal diseases; 17 articles were found with the search. After checking manually, results of 13 original articles were further reviewed. Reviewed articles are listed in **Table 4**. Most of the listed articles studied the diagnostic utility of saliva or GCF MMP-8 to differentiate periodontitis from healthy and/or gingivitis sites but also a few longitudinal studies were found that had been designed to study the disease progression and the predictive utility of MMP-8, the therapeutic response in MMP-8 levels, and in one study the combination of different salivary biomarkers were analyzed in experimental gingivitis (**Table 4**).

In all listed articles, increased GCF or saliva MMP-8 associated significantly with periodontitis. Reported AUC varied from 0.633 to 0.806, suggesting a moderate performance of the test (Fischer et al. 2003). Only one study reported AUC or odds ratio for smokers and non-smokers separately and showed better diagnostic performance for the non-smokers (AUC NS 0.81 vs. S 0.63) (Gürsoy et al. 2010a). Kinney et al. (2014) compared and combined clinical measures, microbial markers, and host-response biomarkers. GCF biomarkers provided a high specificity but a low sensitivity to predict disease progression. Conversely, salivary biomarkers demonstrated a high sensitivity but a low specificity (Kinney et al. 2014). The sensitivity and specificity were more in balance and reached to 70 and 71% by combining the salivary, GCF, the clinical, and the bacterial measures. Within the same data, salivary MMP-8 had 69% sensitivity and 70% specificity (AUC 0.75) to differentiate periodontitis patients from patients without periodontitis and the combination of MMP-8, IL-1 β , *T. denticola* and *P. intermedia* could provide AUC up to a high accuracy of 0.93 (Ramseier et al. 2009).

As a conclusion from reviewed studies of GCF MMP-8, there is a clear association between increased MMP-8 levels and disease severity, and GCF MMP-8 can differentiate between healthy and periodontitis sites. Results indicating the difference between gingivitis and periodontitis are more variable but there is also a trend toward higher GCF MMP-8 in periodontitis sites compared to gingivitis, as was concluded also by Kinane et al. (2011). There is a clear decrease in GCF MMP-8 levels in most of the studies after successful periodontal treatment and the decrease is more probable with the adjunctive LDD treatment.

Studies comparing different biomarkers highlight MMP-8 repeatedly, together with IL-1 β , MPO, and elastase as the best performing diagnostic marker (Uitto et al. 2003, Hernandez et al. 2010, Kinane et al. 2011, Kinney et al. 2014). In addition, RANKL, OPG and ICTP, related to bone resorption and metabolism, can have utility for periodontal POC diagnostics (Reinhardt et al. 2010, Kinane et al. 2011). However, there is a large variability in detected GCF MMP-8 levels at both healthy and diseased periodontal sites. Especially low GCF MMP-8 levels at diseased periodontitis sites can overlap with levels detected from gingivitis or healthy sites producing false negatives and decreasing the specificity of diagnostic MMP-8 screening tests. Similarly, gingivitis sites can occasionally express relatively high GCF MMP-8 levels producing false positives (Mäntylä et al. 2003, Teles et al. 2009, Teles et al. 2010, Kinney et al. 2014)

Many studies also found no significant change in GCF MMP-8 levels after treatment, which indicates variability in GCF MMP-8 response patterns after treatment (**Table 1**), and also possibly variability between different MMP-8 detection methods (Leppilahti et al. 2011)

Kinane et al. (2011, Consensus of the Seventh European Workshop on Periodontology) reviewed whether there are biomarkers fulfilling the above described five categories for the diagnostic biomarkers. They concluded that there is no biomarker available to distinguish those who are susceptible to periodontitis. For *diagnostic* purposes, there is some evidence for MMP-8 and MPO to distinguish between gingivitis and periodontitis, and RANKL/OPG system-related markers. ICPT, prostaglandin E2, β glucuronidase, oncostatin M, cathepsin B, and cathepsin K could also be potential markers for diagnostic purposes. However, there is no marker available, which could differentiate between chronic and aggressive periodontitis. No biomarker could be found to provide this *prognostic* utility, but “there is limited evidence” for certain molecules to

be able to *predict* future disease progression. The response to periodontal treatment can be seen as decreased levels of many inflammatory markers and could be used for *therapeutic* purposes. (Kinane et al. 2011)

Table 3. Studies comparing GCF MMP-8 levels between the progressive/active and the stable sites in longitudinal studies

Author	Author	N of patients (sites)	Difference between active and stable sites	MMP-8 detection method
Lee et al.	1995	58	+	Collagenase activity & WB
Mancini et al.	1999	163	+	Biotinylated-collagen assay
Reinhardt et al.	2010	128	+	WB
Hernandez et al.	2010	(50)	(-)	IFMA
Kinney et al.	2014	100	+	Cytokine Array Raybiotech

Table 4. Summary of articles studying the diagnostic utility of oral fluid MMP-8

Author	Year	N of patients (sites)	Longitudinal studies/ Clinical trials			Diagnostic case-control studies**			
			Sample	Disease progression	Therapeutic response	Experimental gingivitis	P vs. H	P vs. no P	Sensitivity
Kim et al.	2014	314	GCF (pooled)				+		70.4 sensitivity and 85.8 specificity, c- statistics 0.844 (0.793-0.895) for (combination of MMP-8, -9 and -13.)*
Kimney et al.	2014	100	GCF (pooled)	+					23 (13-38)% 95 (85- 99)% (for combination of biomarkers)
Nwhator et al.	2014	76	Saliva				+		60%
Gursoy et al.	2013	230	Saliva				+		55-72% (GP, minor LP, moderate LP)
Ebersole et al.	2013	80	Saliva				+		80%
Lee et al.	2012	30	Saliva		+				AUC 0.84-0.86/ OR 12.1 (composite of markers incl. MMP-8)
Leppilahti et al.	2011	214	Oral rinse				+		AUC 0.70% (0.59- 0.81)
Gürsoy et al.	2011	165	Saliva				+		49/82 83/49 (The cutoff level at the II or III tertiles)
Sexton et al.	2011	68	Saliva		+				AUC: 0.70 (16wk), 0.72 (28wk)
Reinhardt et al.	2010	128	Saliva	+			+		OR 1.5, p = 0.02
Gürsoy et al.	2010	165	Saliva						NS: AUC 0.81, p<0.001 S: AUC 0.63, non-significant
Ramseier et al.	2009	100	Saliva				+		69% 70%
Mäntylä et al.	2003	29(207)	GCF				+		70% (P vs H) 100% (P vs. H) 70% (P vs no P) 89% (P vs. no P)

*Logistic regression analysis was used with multiple clinical parameters (Age, sex, income, smoking, drinking, metabolic syndrome, and biomarkers). MMP-8 was the only significant biomarker as positive indicator in the final best fitting model.

** Periodontitis (P), Healthy (H)

2. AIMS AND HYPOTHESIS OF THE STUDY

MMP-8 is a promising oral fluid biomarker and an option for periodontal point-of-care diagnostics. Oral fluid MMP-8 levels have been reported to associate with the severity of periodontal diseases and clearly decrease after periodontal treatments. However, there has been a quite large variability in the oral fluid MMP-8 levels, which complicates diagnostic interpretations. Large variability can be caused via methodological, a host-related constitutional, or an environmental reason, manifested as variability between and within patients and periodontal sites. The general aim of this study is to evaluate the utility of GCF MMP-8 at the site-level periodontal POC diagnostics and to define different methodological and host-related reasons for the large statistical variability in detected GCF MMP-8 levels. The study hypotheses are: first, Analyses of GCF MMP-8 levels can be utilized in periodontal POC diagnostics; second, there is variability in detection levels between methods based on different MMP-8 antibodies.

The specific aims of this study are:

1. To compare the laboratory and point-of-care detection methods for measuring GCF MMP-8 levels (I, II).
2. To compare cross-sectional correlations between GCF, MMP-8 and other biomarkers (MMP-13, -14, MPO, Azurocidin, CXCL-5) in healthy, gingivitis, and chronic periodontitis sites (II)
3. To study patient effect on detected MMP-8 levels in site-level GCF study (II, III).
4. To study longitudinal variability by exploring different site- level GCF MMP-8 response patterns after non-surgical periodontal treatment in smoking and non-smoking chronic (III, IV) and aggressive periodontitis (IV) patients with cluster analysis
5. To characterize the clinically stable and unstable periodontitis sites and different site-level clinical treatment outcomes during the maintenance treatment period (I, III, IV) for testing the GCF MMP-8 diagnostic and prognostic utility.
6. To study the site-level diagnostic utility of GCF MMP-8 levels:
 - i. Cross-sectional differentiation between healthy or gingivitis sites and periodontitis (II)
 - ii. Prognostic utility to identify different treatment outcomes (III, IV)
 - iii. Therapeutic utility for quantitative monitoring of treatment outcomes (III, IV).

3. MATERIAL AND METHODS

3.1. Patients and periodontal sites

Study I

Inclusion criteria:

1. Moderate to severe chronic periodontitis diagnosed according to the criteria of the 1999 International World Workshop.
2. At least 20 teeth, at least five sites ≥ 4 mm PD and radiographic bone loss.

Six untreated moderate to severe chronic-periodontitis patients, two periodontally healthy and two gingivitis control patients were selected from the larger scale longitudinal study arranged in the clinic of the Institute of Dentistry, University of Helsinki, Finland in 1998-2000 (Mäntylä et al. 2003, 2006). One representative periodontal site was chosen for GCF sampling from each periodontitis patient. GCF samples were collected and clinical parameters recorded at baseline, at the post-treatment control visit approximately 1 month after the treatment, and at the maintenance treatment visits at two-month intervals during the 12 month follow-up. In total, 45 GCF samples were collected from periodontitis patients at 7 to 8 follow-up visits; 38 GCF samples from 2 periodontally healthy (20 samples) and two gingivitis (18 samples) control patients were collected once at baseline. The baseline characteristics of studied sites are presented in the original article (I, Table 1).

Study II

Inclusion criteria:

1. Moderate to severe chronic periodontitis patients according to the criteria of the 1999 International World Workshop.
2. At least 14 teeth, including at least 10 posterior teeth. At least 5 sites with PD ≥ 5 mm and CAL ≥ 3 mm, and detectable bone loss in radiographs.
3. Criteria for gingivitis control patients were inflamed gingiva and BOP with no signs of attachment loss. Criteria for healthy controls were PD < 3 mm and no CAL or BOP.

Eight untreated patients with moderate to severe chronic periodontitis, 9 healthy, and 6 gingivitis control patients took part to this cross sectional study. Patients were recruited from the Center of Diagnostics and Treatment of Northern Metropolitan Health Services, Santiago, Chile in 2010. A total of 58 GCF samples were collected from all patients [20 healthy (from 1 to 3 sites/patient), 19 gingivitis (from 1 to 4 sites/ patient), 19 CP (from 1 to 4 sites/patient)]. Patient demographics are presented in the original article (II, Table 1)

Study III

Inclusion criteria:

1. Moderate to severe CP diagnosed according the criteria of the 1999 International World Workshop.
2. At least 20 teeth, at least five sites \geq 4mm PD, and radiographic bone loss.

Altogether 15 untreated patients with moderate to chronic periodontitis were recruited from the clinic of the Institute of Dentistry, University of Helsinki in 1998-2000. For each patient, 5 to 7 periodontal sites were monitored throughout this longitudinal study. All patients received the SRP treatment at the beginning and the maintenance treatments in the two months interval. The longitudinal follow-up included the baseline visit, the post-treatment visit, and 4 to 6 maintenance visits with clinical measurements and GCF sampling. The study data included 742 GCF samples from 97 periodontal sites. There is an error in the text of the attached original article (III, Material and Methods, p. 251) related to the number of smokers and non-smokers and males and females. The correct baseline characteristics of studied patients and sites are presented in **Table 5**.

Study IV

Inclusion criteria: The aim of the inclusion criteria was to ensure that all GCF samples were collected, and MMP-8 levels determined, with the same method. The original study designs also had to be similar (clinical trials with SRP) to explore differences in longitudinal GCF MMP-8 response patterns in the different datasets. Thus, the following inclusion criteria were defined:

1. Diagnosed periodontitis (CP or AgP).
2. GCF was sampled at all sites with the same method. A paper strip is held for 30 seconds in the gingival crevice.
3. The same monoclonal antibody and the IFMA method was used for the detection of MMP-8.
4. Longitudinal study design (clinical trials) including the conservative periodontal treatment (SRP) for previously untreated periodontitis patients, followed by maintenance visits with regular biofilm removal and oral hygienic instructions. The length of the maintenance was required to be at least 6 months.

Four independent longitudinal datasets met the criteria. Three datasets comprise Turkish patients recruited from the Ege University, İzmir, Turkey (Department of Periodontology, School of Dentistry). One dataset with generalized aggressive periodontitis patients (G-AgP) and two dataset with chronic periodontitis patients. One dataset comprised Finnish chronic periodontitis patients recruited from the Institute of Dentistry, University of Helsinki. From the Finnish dataset, 74 periodontitis sites were included from 15 patients (from 2 to 7 sites/ patient).

From the Turkish datasets altogether 32 G-AgP and 52 CP patients were included. In the Turkish data, only one site/patient was sampled for GCF. Clinical measurements and GCF sampling were done at baseline, at the post-treatment visit 1 month after SRP treatment, and at the maintenance-treatment visits 3 and 6 months from the baseline.

Original inclusion criteria of individual included datasets in study IV were as follows:

Data 1

Severe, generalized aggressive periodontitis patients, a clinical trial with SRP + azithromycin or placebo, Izmir, Turkey 2004-2006:

1. Severe generalized aggressive periodontitis.
2. At least 16 teeth. >30 sites with ≥ 5 mm CAL and ≥ 2 sites with a PD ≥ 6 mm in each quadrant that had BOP (Emingil et al. 2012).

Data 2

Severe generalized chronic periodontitis patients, clinical trial with SRP+ azithromycin or placebo, Izmir, Turkey 2004-2006):

1. Diagnosed severe generalized chronic periodontitis.
2. At least 16 teeth, CAL level of ≥ 5 mm and PD of ≥ 6 mm on ≥ 8 teeth (≥ 3 other than central incisors or first molars); 30% or more bone loss of the root length in radiographs (Han et al. 2012a).

Data 3

Moderate and severe chronic periodontitis patients, clinical trial with SRP + LDD or placebo, Izmir, Turkey 1999-2001):

1. Diagnosed moderate to severe chronic periodontitis.
2. At least 14 teeth, at least eight pockets with ≥ 5 mm PD and ≥ 4 mm CAL, and radiographic evidence of moderate to severe chronic periodontitis. (Emingil et al. 2004)

Data 4

Moderate and severe chronic periodontitis patients, clinical trial with SRP, Helsinki, Finland 1998-2000):

1. Diagnosed moderate to severe CP.
2. At least 20 teeth, at least five sites ≥ 4 mm PD and radiographic bone loss. (Mäntylä et al. 2006)

All studies/data (I, II, III and IV) in this thesis had similar common exclusion criteria:

1. Any history of severe medical disorder or systemic disease
2. Pregnancy
3. Received antibiotics or other medicines or periodontal treatment within the past 4-6 months.

In the Turkish clinical trials (IV) heavy smokers were excluded (smoking >10 cigarettes per day). In addition, hypersensitivity to tetracycline, doxycycline, or any type of

macrolide was an exclusion criteria in the Turkish trials (IV) due to the adjunctive medication with LDD or azithromycin.

The study protocols were approved by the ethics committee of the Institute of Dentistry, University of Helsinki, by the Ethics Committee of the Ege University School of Medicine, Izmir, Turkey, and by the institutional review board from the Faculty of Dentistry, University of Chile. Study patients gave informed consent to participate in the study.

Table 5. Baseline demographics and clinical parameters in studies III and IV.

	Study III	Study IV			
		Data 1	Data 2	Data 3	Data 4
Demographics					
Disease type	CP	G-AgP	CP	CP	CP
N of patients	15	32	28	24	15
N of sites	97	32	28	24	74
Visits	6- 8	4	4	3	4
GCF samples	742	128	112	72	296
Gender, patients (sites)					
Males	5 (32)	17	18	15	5 (25)
Females	10 (65)	15	10	9	10 (49)
Smoking, patients (sites)					
Non- smokers	5 (30)	17	16	14	5 (23)
Smokers	10 (67)	13	12	10	10 (51)
Medication, patients (sites)					
None	15 (97)	16	14	12	15 (74)
Azithromycin		16	14		
LDD				12	
Age, mean (95% CI)	43 (41-45)	29 (27-31)	46 (44-48)	46 (43-49)	43 (41-46)
Clinical parameters					
CAL, mean (95% CI)					
Non- smokers	5.9 (4.7-7.0)	7.7 (7.0-8.5)	7.8 (6.9-8.6)	7.7 (6.8-8.5)	7.1 (6.0-8.1)
Smokers	4.9 (4.4-5.5)	8.2 (7.2-9.1)	7.4 (6.6-8.2)	7.8 (6.3-9.3)	5.7 (5.2-6.3)
PD, mean (95% CI)					
Non- smokers	6.1 (5.6-6.6)	7.1 (6.4- 7.8)	6.2 (5.7-6.7)	6.8 (6.2-7.4)	6.5 (6.0-7.1)
Smokers	5.5 (5.3-5.7)	7.5 (6.6-8.4)	5.8 (5.3-6.4)	6.9 (6.3-7.4)	5.8 (5.6-6.1)
BOP% all teeth, mean (95% CI)					
Non- smokers	data not available	81 (72-90)	75 (64-85)	75 (62-88)	data not available
Smokers	data not available	75 (67-85)	57 (43-71)	73 (63-82)	data not available
BOP% sampled sites, mean (95% CI)					
Non- smokers	100	100	100	100	100
Smokers	73 (62-84)	100	100	90 (67-100)	71 (58-84)

3.2. Clinical periodontal examinations and treatments

Probing depth (PD), clinical attachment loss (CAL), bleeding on probing (BOP), and visible plaque (VP) was recorded from six sites around the teeth. In each sub-study all the clinical examinations, the GCF samplings, and the treatments were done by the same calibrated periodontologist (I: P.M., II: J. G. III: P.M. IV: P.M & B.H.). All the patients received scaling and root planing treatment, in addition to oral hygiene instructions after baseline examinations and maintenance treatments according to the study plan. Study IV included data from the clinical trials testing the effect of low dose doxycycline (LDD) or azithromycin adjunctive to the conventional SRP treatment, as half of the patients received the medication and the other half placebo.

3.3. GCF Sampling

Before GCF sampling, teeth were cleaned and the supragingival plaque was removed with a sterile curette; the surfaces of sampling sites were gently air dried, and isolated by cotton rolls. A filter-paper sampling strip was gently placed into the gingival crevice for 30 seconds, avoiding any bleeding from the gingiva. After the GCF sampling proteins in the paper strips were eluted into the buffer solution and frozen for the storage and transportation (samples from Helsinki) or strips were frozen directly in the test tubes and proteins eluted into the buffer solution afterwards in the laboratory (Turkish and Chilean data). The elution buffer contained 50 mM Tris-HCl pH 7.5, 0.2 M NaCl, 5 mM CaCl₂, and 0.01% Tritón X-100 (II) or HEPES pH 7.4 (Mäntylä et al. 2003; I, III, IV) or 20mM Tris HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl₂, 50µM ZnCl₂, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg/l DTPA (Hanemaaijer 1997, Turkish data, IV). No significant difference in MMP-8 levels was evident between assay runs with different assay buffers (non-published data). Both HEPES and Tris- HCl are suitable elution buffers in MMP-8 immunoassays (Hanemaaijer et al. 1997, Mäntylä et al. 2003, 2006), but if MMP-8 activities are also to be measured, then Tris-HCl, ph 7.5-7.8 buffers are recommended (Golub et al. 2008, Reinhardt et al. 2010).

3.4. Biomarker detection methods

3.4.1. MMP-8 time-resolved immunofluorometric assay (IFMA)

MMP-8 levels were measured with the quantitative time-resolved immunofluorometric assay (IFMA, Medix Biochemica Ab Oy, Finland) as described thoroughly by Hanemaaijer et al. (1997). The monoclonal MMP-8-specific antibodies 8708 and 8706 were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labeled using europium-chelate. The assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl₂, 50 mM ZnCl₂, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg/l DTPA. Samples were diluted in the assay buffer and incubated for 1 h, followed by incubation for 1 h with the tracer antibody. The enhancement solution was added, and after 5 min fluorescence was measured using a fluorometer. (Hanemaajer et al. 1997)

3.4.2. POC MMP-8 detection methods: dentoAnalyzer® and stick-test

The dentoAnalyzer® (DentoGnostics GmbH, Germany) is a portable bench-top device design to measure biomarker levels from oral fluids. It automatically runs the assay process, including liquid handling as well as displaying final outcomes calculated by a software program. The key component is a cartridge consisting of a liquid-handling module including all needed reagents for immunological reactions like a clinical sample, conjugate, wash buffers, and substrate and a reaction chamber containing six filters with positive and negative controls, where the immunological reactions take place (Munjal et al. 2007a). Two antibodies detecting specific epitopes of the antigens are used in a sandwich-based immunoassay technology known as antibody immuno columnor analytical process (ABICAB), which is based on an immunoaffinity filter design using flow through solid phase filters with extremely high binding potential (Hartmann et al. 1993, Stove et al. 1995, Cavuslu et al. 2003, Meyer et al. 2007). DentoAnalyzer® is based on the same monoclonal antibodies as the IFMA method. Methodology for the dentoAnalyzer® is described in more detail by Munjal et al. (2007).

The MMP-8 specific immunochromatographic dip-stick (Medix Biochemica Ab Oy, Finland) is a qualitative POC test. The test result is provided in 5 min and is estimated by eye as positive or negative (Mäntylä et al. 2003, 2006). The test involves two monoclonal

antibodies to human MMP-8 (the same as in IFMA and dentoAnalyzer®, 8708 and 8706). One is bound to blue latex particles to act as the detecting label. The other antibody is immobilized on a carrier membrane to catch label particles and indicate a positive result. When the eluted GCF sample liquid is placed in the dip area of the stick, liquid is absorbed and begins to flow up the dip- stick. If there is MMP-8 in the sample it binds to the antibody attached to the latex particles. The antibody-MMP-8 particles are carried by the liquid flow and detected by the catching antibody. A positive test result, if the cutoff level is exceeded, will appear as a blue line. A second blue line confirms the correct performance of the test. The dip-stick test is based on the same monoclonal antibodies as the IFMA analysis (8708 and 8706).

3.4.3. MMP-8 ELISA

The commercially available MMP-8 ELISA analysis was conducted according the manufacturer's instructions (Biotrak ELISA system, GE Healthcare, Amersham, Berkshire, UK). Levels were obtained from a standard curve and expressed as ng per ml of eluted GCF.

3.4.4. MMP-8 western blot

The molecular forms of MMP-8 were detected by using the modified enhanced chemiluminescence (ECL) Western blotting kit according to the protocol recommended by the manufacturer (GE Healthcare, Amersham, UK) as described earlier (Hernandez et al. 2010). Briefly, the GFC samples were mixed with Laemmli's buffer without any reducing reagents and heated for 5 min, followed by protein separation with 11% sodium dodecyl sulphate (SDS)-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes (Protran®, Whatman GmbH, Dassel, Germany). Non-specific binding was blocked with 5% milk powder (Valio Ltd., Helsinki, Finland) in TBST buffer (10 mM Tris-HCl, pH 7.5, containing 22 mM NaCl and 0.05% Triton-X) for 1 h. The membranes were incubated with the monoclonal primary antibody, anti-8708 (Medix Biochemica Ab Oy, Finland) overnight, followed by the horseradish peroxidase-linked secondary antibody (GE Healthcare, Buckinghamshire, UK) for 1 h. The membranes were washed 4 times for 15 min in TBST between each step. The proteins were visualized using the ECL system and scanned and analyzed using GS-700

Imaging Densitometer Scanner (Bio-Rad, Hercules, CA, USA) and the Bio-Rad Quantity One program.

3.4.5. Determination of MMP-13, MMP-14, TIMP-1, MPO, Azurocidin, and CXCL5 Levels from GCF

Total levels and the portion of endogenous active MMP-13 and MMP-14 from eluted GCF were defined separately with or without adding aminophenylmercuriacetate (APMA, Sigma, St.Louis, MO, USA) into aliquots. The MMP-13 analysis was performed by using the Human Active MMP-13 Fluorokine E kit (R&D Systems, Inc. Minneapolis, USA), and MMP-14 analysis with the MMP-14 Biotrak activity assay system (GE Healthcare, Amersham), according to the manufacturer's recommendations. Levels were obtained by interpolating from a standard curve and expressed ng per ml of eluted GCF (Hernandez et al. 2006, Hernandez et al. 2010).

Total levels of TIMP-1 (Biotrak ELISA system, GE Healthcare, Amersham, Slough, Berkshire, UK), MPO (Immundiagnostik, AG, Bensheim, Germany), azurocidin (Cusabio Biotech Co. Ltd, Wuhan, China) and CXCL5 (R&D Systems, Minneapolis, MN, USA), were determined with the commercial ELISA kits according manufacturer's instructions. Levels were obtained by interpolating from standard curves.

3.5. Statistical analysis

3.5.1. Statistical comparisons of study groups

Cross-sectional MMP-8 distributions appeared to be ubiquitously right skewed in all data sets in this thesis (I, II, III, IV) as has also been described elsewhere (Teles et al. 2009, Kraft-Neumärker et al. 2012, Kinney et al. 2014). Non-parametric methods, Wilcoxon signed rank test (for dependent variables), and Mann-Whitney U test and Kruskal-Wallis (for independent variables) were used for statistical comparisons between the study groups if the data normalization was not done (I, III). Non-parametric Spearman correlations were favored for skewed data. In study IV, mean and 95% confidence intervals of MMP-8 levels and clinical parameters were reported descriptively, as was also done for study III parameters in the summary part of the thesis to make the comparisons between studies III and IV easier, although in the original article of study

III, the MMP-8 levels and clinical parameters were given in medians and respective IQR was reported. In study II, log normalization was conducted on the MMP-8 levels and the other biomarkers not obeying the normal distribution to legitimize the use of the t-test, the ANOVA F test, and the regression model described below. Pearson chi-squared test was used in cross-tabulations. Statistical programs SPSS (IBM, NY, USA) and R (<http://www.R-project.org>) were used to conduct the analysis.

3.5.2. Data normalization of the pooled data

In study IV, data was pooled from four independent datasets. The data inclusion criteria were aimed to ensure that the GCF sampling and the detection of GCF MMP-8 levels were performed with the same method. However, there are always discrepancies between assay runs in immunological detection methods, which can be seen as scalar differences (Holzel1991, Elshal & McCoy2006). Thus, the data in study IV was normalized into the same scale (from 0 to 1). The normalized level can be interpreted as a percentage measure from the population maximum. Before the normalization, MMP-8 distribution types were assessed to be similar in all the datasets and the procedure was determined to be eligible by analysing the distributions with quantile to quantile (Q-Q) plots (SPSS). The gamma distribution gave the best fit with GCF MMP-8 distributions in the data (Q-Q plots, **Figure 2**, **Figure 3**, non-published data). The Q-Q plot is a graphical method to compare distributions with each other. In this context, distributions from different datasets (empirical data) were compared to different test distributions (gamma, exponential, normal, log-normal etc.).

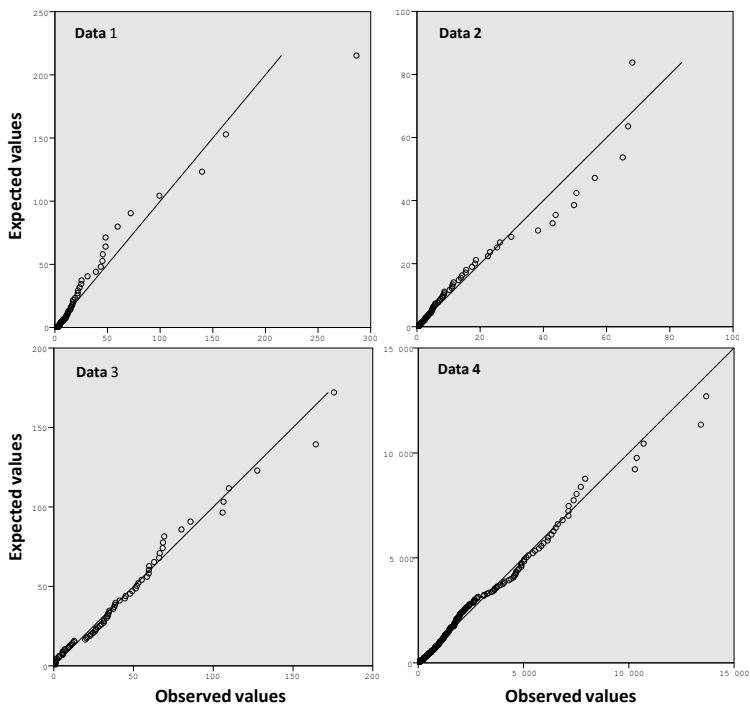


Figure 2. Q-Q plots of the original GCF MMP-8 levels (observed) compared to the gamma test distribution (expected) in four different datasets of the study IV.

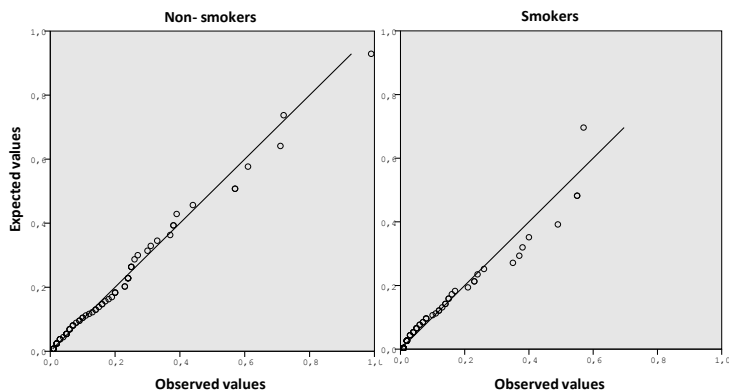


Figure 3. Gamma Q-Q plots of the normalized GCF MMP-8 levels in study IV cross-sectionally at the baseline; A) non- smokers and B) smokers defined separately. Estimated shape and scale parameters of gamma distribution for the smokers: 0.45 and 4.97; and the non-smokers: 0.91 and 4.89 (non-published data).

3.5.3. Generalized mixed regression model

A generalized mixed regression model was used to study the patient effect in the site-level GCF study (II). The model allows hierarchical dependence and the clustering of samples (patient -> sites) in the data. The analyzed biomarker level was considered to be an outcome measure, the periodontal health status as an explaining fixed effect, and the patient as a random effect. The analysis were performed using the packages “nlme” and “lmerTest” in R (<http://www.R-project.org/>). An ANOVA F test and a post hoc t-test with a Bonferroni correction for an alpha level of 0.05 were used for comparing sites belonging to the healthy, the gingivitis, and the periodontitis groups.

3.5.4. Receiver operating characteristics (ROC) analysis

ROC analysis was conducted to evaluate the diagnostic utility of MMP-8 levels in the cross-sectional setting (II) and to search the most optimal cutoff levels for the analysis of prognostic utility (III and IV). In study II, a bayesian analyzing method was used to take the clustering of data into account. Three MCMC chains were used, and the burn-in phase was extended until convergence was met, as determined by the Gelman–Rubin statistic. The posterior distributions were calculated from a total of 10,000 samples. SPSS (III and IV) and the software JAGS, using R (II) were used to run the analysis.

3.5.5. Cluster analysis

A cluster analysis was used to explore the longitudinal site-specific MMP-8 response patterns (III and IV). For the analysis, the established k means method was employed with the correlation (Pearson) distance measure and 1000 replicate runs. The correlation (Pearson) distance measure, maximizing the correlation between longitudinal sections (response patterns) of different periodontal sites within the cluster, was used to emphasize the shape of the longitudinal response patterns (MMP-8 or CAL change.).

Smokers and non-smokers were analyzed separately. The site level MMP-8 response patterns were clustered into two ($k = 2$, III) or into four groups ($k = 4$, IV) depending on the study size. Also, clinical treatment outcomes were categorized by using the same cluster analysis method in study III. Sites were clustered according to CAL change during

the maintenance period into two groups ($k = 2$). Statistics Toolbox of MATLAB program (MathWorks, Natick, MA) was used to conduct the cluster analysis.

3.5.6. Analysis of prognostic utility

The prognostic utility of GCF MMP-8 levels was tested by first defining the most optimal cutoff levels by maximizing the sum of sensitivity and specificity (the Youden index) at baseline (III and IV) and the maintenance period (IV) from ROC analysis and then cross tabulating the MMP-8 test positives and negatives against the categorical treatment outcomes (the weak/compromised or the successful treatment outcome, based on CAL change). Pre- and post-test probability, negative and positive predictive values, and likelihood ratios were calculated from the cross-tabulations to evaluate the prognostic utility of MMP-8 levels. The prevalence of compromised clinical treatment outcomes was used as the pre-test probability at baseline. Statistical variability was indicated by 95% confidence intervals. In the original article of study III, the term “weak” was used instead of “compromised” treatment outcome. However, only the term “compromised”, which was used in study IV, is used for clarity in the summary part of this thesis, because it describes the clinical outcome better (see also 4.3. clinical treatment outcomes in longitudinal studies).

4. RESULTS

4.1. Cross-sectional variability in MMP-8 levels

4.1.1. Differences between detection methods and comparison of POC methods

IFMA method correlated well with dentoAnalyzer® (correlation coefficient 0.95; Spearman correlation) and results of the qualitative dip-stick test was well in line with the quantitative IFMA and dentoAnalyzer® methods (I). The commercial MMP-8 ELISA did not correlate significantly ($p > 0.05$) with the IFMA, dentoAnalyzer® or dip-stick (I). On the other hand, there was significant correlation between the MMP-8 levels of IFMA and the commercial ELISA in study II (all sites: $r = 0.87$, $p < 0.001$, periodontitis sites: $r = 0.91$, $p < 0.05$).

More detailed analysis of the GCF MMP-8 detection methods, commercial ELISA, IFMA, and the POC methods revealed that with the low GCF MMP-8 levels, the commercial ELISA, IFMA, and DentoAnalyzer® levels were at the same levels but at higher levels the commercial ELISA did not follow along with IFMA, as was the case with dentoAnalyzer® or the stick test in the study I (**Table 6**).

There were also differences between IFMA and ELISA in the correlation profile of MMP-8 and the other studied biomarkers. MMP-8 IFMA also had better discriminatory properties between periodontitis and gingivitis or healthy sites compared to the MMP-8 ELISA method (II, Table 3 and 4).

There was a significant decrease in MMP-8 levels after SRP treatment and during the maintenance period if MMP-8 was measured with IFMA or dentoAnalyzer®, instead the decrease was not statistically significant with the ELISA (**Table 6**).

Table 6. Comparison of different MMP-8 detection methods in study I.

Detection method, mean (range) ng/ml	Baseline	After treatment	p value
IFMA	5 900 (1 690- 13 400)	2 180 (457- 6 420)	0.028
DentoAnalyzer	213 (17- 311)	61 (5- 311)	0.043
Commercial ELISA	15.9 (1.8- 36.7)	10.5 (1.2- 17.6)	>0.05

	IFMA < 1000 *	IFMA 1000- 4000	IFMA > 4000	
IFMA	487 (35- 877)	2 080 (1 200- 3 610)	9 250 (4 260- 18 100)	<0.001
DentoAnalyzer	5.1 (1- 13)	57.3 (7- 222)	309 (290- 311)	<0.001
Commercial ELISA	6.7 (2- 17.6)	8 (1.8- 17.5)	9.7 (0.6- 36.6)	>0.05
Test stick	11.8	43.8	100	<0.001
% of pos. results				

* MMP-8 levels detected by the IFMA method are categorized into three category IFMA <1000 ng/ml, 1000-4000 ng/ml and >4000 ng/ml, respectively, for comparison with other detection methods in the lower cross tabulation.

4.1.2. Differences between healthy, gingivitis and periodontitis sites in MMP-8 isoforms

The activated, 55 kDa MMP-8 immunoreactive species were observed only in GCF samples from periodontitis sites in the western immune blot analysis based on the same monoclonal antibody (anti-8708, Medix Biochemica Ab Oy, Finland) as IFMA, dentoAnalyzer® or dip-stick test (I). On the contrary, gingivitis sites had mainly the latent (proform) 65 kDa species; as in healthy sites hardly any immunoreactivity was detected. The high molecular weight complexes (>100 kDa) and the fragmented species (30-40 kDa) were detected in both gingivitis and periodontitis sites (I, Figure 5).

4.1.3. Cross sectional differences between healthy, gingivitis and periodontitis sites in GCF MMP-8 levels

Statistical comparisons between chronic periodontitis and healthy or gingivitis sites were performed in study II. Mean (95% CI) GCF IFMA MMP-8 levels in healthy, gingivitis and periodontitis sites were 40 (13-67), 115 (54-176), and 367 (250-484) ng/ml, respectively. The difference between periodontitis and gingivitis ($p = 0.003$) or healthy ($p < 0.001$) sites was significant. In ANOVA, the variance between study groups (healthy, gingivitis, chronic periodontitis) explained 54% of the whole variance as within-group variance explained 46%.

4.1.4. Correlations between MMP-8 and the other GCF biomarkers

In periodontitis sites (II), there was a high correlation between the IFMA MMP-8 levels and MPO ($r = 0.95$, $p < 0.001$), and significant correlation between IFMA MMP-8 and MMP-13 levels (total levels: $r = 0.55$ and active: $r = 0.59$). In subjects with healthy periodontium, IFMA MMP-8 correlated significantly with azurocidin. In the gingivitis group, no significant correlations between MMP-8 IFMA and other biomarkers were found as MMP-8 ELISA levels correlated with MMP-14. Correlation coefficients and respective p-values between the different biomarkers are presented thoroughly in the original article (II).

4.1.5. The patient effect on site level GCF MMP-8 levels

In site-level studies, there can be patient-related clustering of data if multiple sites per patient are included in the study. The patient effect was taken into account with mixed-regression model analysis (II). The patient effect size was approximately 13% from the whole variance explaining part of the “within” variance (46% from the whole variance). However, the (random) patient effect was insufficient to improve the accuracy of the used regression model and thus was discarded in further analysis.

In study III, the longitudinal variability of MMP-8 levels was evaluated. Site-level longitudinal GFC MMP-8 response pattern types were clearly gathered within the same patients (III, table 4). In the smokers, 20 profile-1 sites were clustered into five patients, as the other five patients presented only one profile 1 site. Profile-2 sites were observed in all patients. In the non-smokers sites, profile-3 sites were clustered into three patients (out of 5) as all the non-smokers had profile-4 sites. Different response-pattern types are described in more detailed below, in the section 5.2. “longitudinal GCF response patterns”. (III).

4.1.6. The effect of smoking on GCF MMP-8 variability

The effect of smoking was controlled in studies II, III, and IV. In studies III, and IV, smokers and nonsmokers were stratified separately. In study II, the smoking status could not be stratified due to a lower data size but the number of smoking patients was statistically equal in all groups. There was a clear and statistically-significant difference

in the GCF MMP-8 levels between smoking and the non-smoking patients at baseline (III, IV).

In study III, mean (95% CI) GCF MMP-8 levels were 1360 (810-1920) ng/ml for the smokers and 4440 (3180-5690) ng/ml for the non-smokers at baseline. In study IV, the normalized mean (95% CI) level was 0.09 (0.06- 0.12) in the smokers, and 0.19 (0.14- 0.23) in the non-smokers, corresponding to 1640 (1090-2190) ng/ml and 3460 (2550- 4190) ng/ml in study III.

During the maintenance period (III, IV), non-smokers’ mean GCF MMP-8 levels decreased significantly and retained at lower levels, while the mean levels decreased in the smokers’ at first but then returned nearly to baseline levels (**Figure 4**). The mean MMP-8 levels were approximately at the same level (1000-2000 ng/ml) from 3 to 4 months after SRP (III, IV), and no significant difference between the smokers and non-smokers was detected later on during the maintenance period (**Figure 4**). Different types of MMP-8 response patterns, which could be explored via cluster analysis as described below, were evident for both non-smokers and smokers.

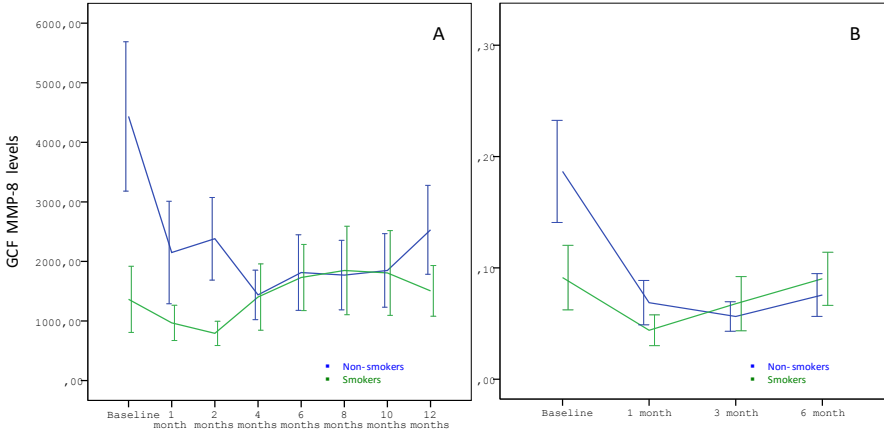


Figure 4 Mean (95% CI) GCF MMP-8 levels in the smokers and the non-smokers at different time points of studies III (A) and IV (B).

4.2. Longitudinal GCF MMP-8 response patterns

In study I, stable and unstable sites were defined according to clinical parameters and compared to MMP-8 levels. In the stable sites, mean CAL and PD levels decreased after SRP treatment together with the MMP-8 levels and both the clinical parameters and the MMP-8 levels retained at low levels during the maintenance period (I, Figure 1). In unstable sites, PD decrease and CAL gain was lost soon after treatments and MMP-8 levels tended to increase or fluctuated during the maintenance period.

In studies III and IV, a similar longitudinal set-up was tested with the larger study sample, and the longitudinal GCF MMP-8 response patterns after SRP treatment were explored with cluster analysis. Smokers and the non-smokers were handled separately. In study III, sites were clustered into two groups, while in study IV, sites could be clustered into four groups due to the larger data. The idea of the cluster analysis with varying numbers of clusters was to determine different response patterns, which are not observable in the overall mean levels (IV, Figure 1).

Both smokers and non-smokers could be clustered into two distinct groups according to their baseline GCF MMP-8 levels and response patterns during the maintenance period. Characteristically, sites with high GCF MMP-8 levels at baseline (the Type I response pattern) presented the most obvious decrease after non-surgical periodontal treatment while sites with low GCF MMP-8 levels at baseline (the Type II pattern) demonstrated increasing levels in relative to baseline during the maintenance period (III, Figure 1; IV, Figure 1). In study III, the number of non-smoker sites and patients was low and the differentiation of patterns was unclear. However, two distinct groups could also be clustered among the non-smokers from the larger data in study IV.

The smokers' sites with type-II response patterns could be differentiated further into high- and low-responding sites according to the relative increase of GCF MMP-8 levels during the maintenance period. In an earlier study, the cutoff at 4000 ng/ml twice or more often during the maintenance period was used for the high-responders (Mäntylä et al. 2006). The sub-group of high-responders among smokers was also explored "agnostically", without any presumptions, as an own group when the sites were clustered into four groups (IV, Figure 1). High-responders continuously displayed exceptionally high MMP-8 levels during the maintenance period. Among the non-smokers, such a distinct group of high-responders could not be differentiated in the cluster analysis, but

there were individual sites with high MMP-8 levels during the maintenance period. The high MMP-8 levels increased the risk for compromised treatment outcome twice, or more often, during the maintenance period in the both smoking and non-smoking patients, as described below.

4.3. Clinical treatment outcomes in longitudinal studies

In studies III and IV, cluster analysis was employed to explore general trends in CAL change during the maintenance period, to form a dichotomous “dummy” variable for testing the utility of GCF MMP-8 levels to give a prognosis for the respective clinical-treatment outcome. Cluster analysis works “agnostically” to explore groups with similar response patterns, whilst also maximizing difference between groups. However, there is no guarantee that the dichotomy produced by the clustering would have any clinical rationality. Thus, results were compared between the cluster analysis and the “consensus definition” of a significant CAL change (2 mm cutoff) at the end of the maintenance period.

In studies III and IV, two distinct groups were explored with cluster analysis in both smokers and non-smokers. The groups were given names ‘compromised’ or ‘successful’ according the trend in CAL change. There was a significant PD decrease after the treatment in most sites in both clusters, but the trend was that CAL change did not follow along with PD change in the group of compromised outcomes (**Figure 5, and 6**). There was a significant difference in CAL change ($p < 0.001$) between the clusters of compromised and successful outcomes during the end of maintenance period (III and IV). Sites with compromised outcomes demonstrated less CAL gain, or there was no significant change in CAL when sites with successful outcome had significant CAL gain near the measures of PD decrease. In the smokers’ sites, the mean CAL gain was near zero in the group of compromised outcomes in the both studies (III and IV) when in the non-smokers the mean CAL gain was between 1-2 mm in the group of compromised outcomes compared to successful outcomes, with 3-4 mm CAL gain at the end of maintenance.

When PD decrease, CAL gain, and gingival recession (GR) are presented together (**Figure 5.**), it appears to be quite obvious that in the sites with the compromised outcome, PD decrease is achieved more through GR, while in the sites with the successful

outcome, PD decrease and CAL gain is achieved through re-attachment of junctional epithelium (III, IV).

When the cluster analysis and the consensus method of 2 mm (Haffajee et al. 1983) are compared it seems that the methods gave surprisingly similar results (**Figure 6**). In study III, there was no significant difference in the mean CAL change whether the weak/compromised treatment outcome was defined with the cluster analysis or by using the cutoff of 2 mm mean CAL gain at the end of the maintenance period (6-12 months mean) (**Figure 6**). Similarly, in study IV, there was no difference among the smokers in the comparison, respectively (cluster analysis vs. CAL gain > 2 mm after 6 months). Nevertheless, a more clinically rational differentiation was achieved by using the 2 mm cutoff compared to cluster analysis in non-smokers, and this approach was used for further analysis in study IV (**Figure 6**).

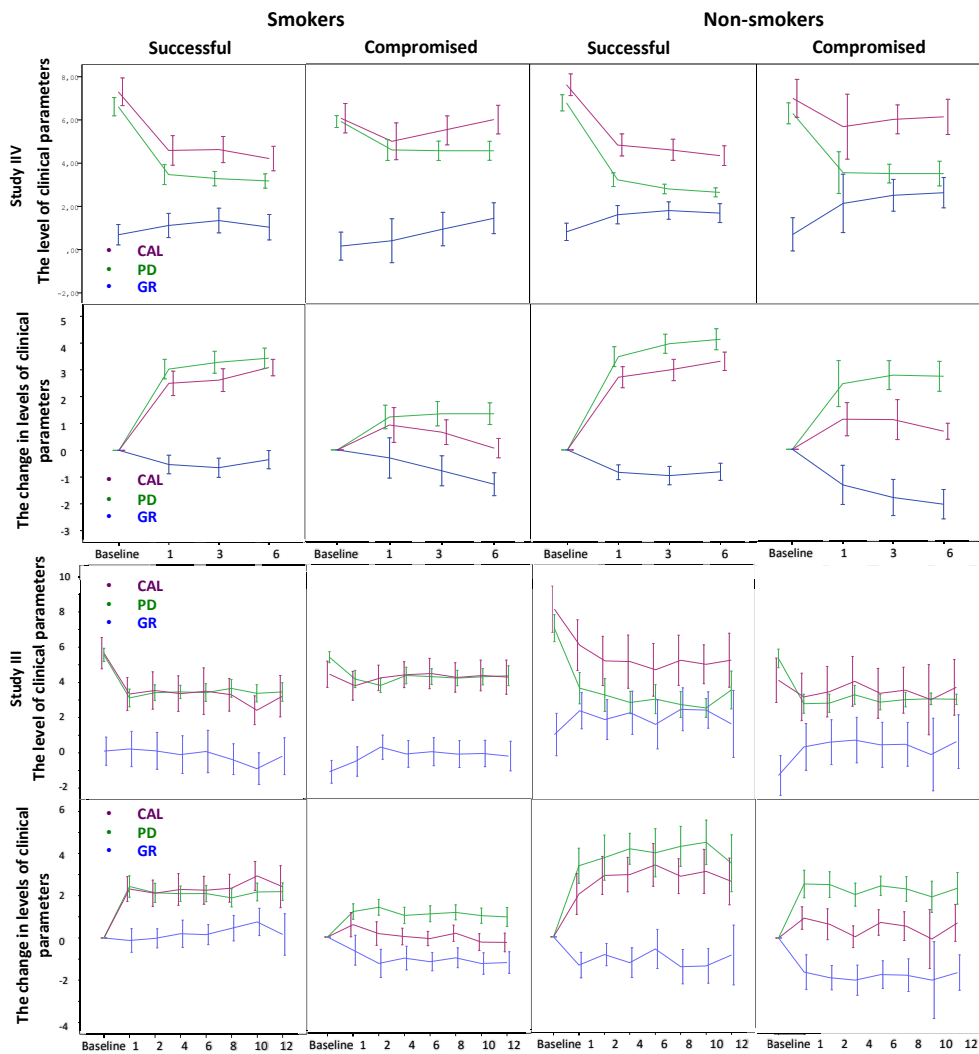


Figure 5. Clinical parameters and their changes in different categorical treatment outcomes (successful and compromised*) at different points in studies III and IV.

*The divergence of PD decrease and CAL gain and larger gingival recession is presented in the groups of compromised treatment outcome. PD decrease and CAL gain are indicated with positive measure/sign and GR with negative sign. GR = gingival recession, PD = pocket depth, CAL = clinical attachment level.

*In study III, the compromised and successful treatment outcome was determined with cluster analysis and in study IV, by the 2 mm cutoff at the end of the maintenance period (6 months).

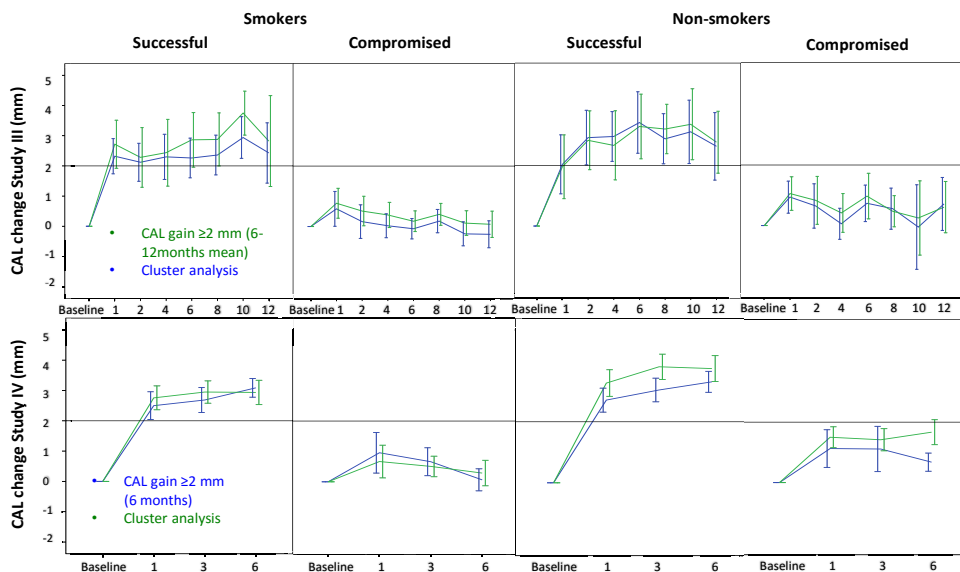


Figure 6. Comparison of trend lines (mean, 95%CI) of categorical treatment outcomes defined either with the cluster analysis or the “consensus” 2 mm cut-off* in the studies III and IV. *In study III the site-specific mean CAL change was calculated from 6-12 months and sites with CAL gain ≥ 2 mm (negative CAL change) was categorized as successful, and < 2 mm as compromised, respectively. In study IV, which comprised less maintenance visits, the 2 mm cutoff was based only on the month six visit.

4.4. Diagnostic and prognostic utility of GCF MMP-8 levels

4.4.1. Diagnostic utility and screening performance of GCF MMP-8 and the other studied biomarkers

GCF MMP-8 (IFMA) levels could clearly discriminate (AUC: 0.97; 95% CI: 0.85-1) between periodontitis sites and gingivitis or healthy sites when the patient effect was taken into account (II). Other biomarkers could also differentiate between periodontitis and healthy sites: MPO (AUC: 0.98; 95% CI: 0.78-1), MMP-13 total (AUC: 0.94; 95% CI: 0.72-1), MMP-14 total (AUC: 0.95; 95% CI: 0.78-1), Azurocidin (AUC: 0.90; 95% CI: 0.71-1). Only MMP-8 and MPO, however, could differentiate significantly ($p < 0.05$) between periodontitis and both gingivitis and healthy sites (II). From the studied biomarkers, CXCL5, MMP-13 active, and MMP-14 active had no significant discriminatory properties between periodontitis and gingivitis or healthy sites (II). Both MMP-8 IFMA and MMP-8

ELISA could discriminate between periodontal health status, but ELISA methods had lower ROC values and larger variability in the AUC score (AUC: 0.90; 95%CI: 0.54–1) compared to IFMA. The most optimal cutoff values, defined from the ROC curves and MMP-8 IFMA cutoffs, had a sensitivity of 0.95 and specificity of 0.94 as MMP-8 ELISA had 0.89 sensitivity and 0.87 specificity, respectively

4.4.2. Prognostic utility of GCF MMP-8 levels

The prognostic utility of GCF MMP-8 levels was analyzed by first determining the optimal cutoff levels to discriminate between the physiological and pathological GCF MMP-8 levels. The cutoffs were evaluated with ROC analysis: The GCF MMP-8 level with the highest discriminatory properties to differentiate between the clustered MMP-8 response patterns, followed by clinical treatment outcomes (**Table 7**).

In study III, two baseline-cutoff levels were defined for smokers, 770 ng/ml was the best level to discriminate between the two MMP-8 response patterns (profile/pattern type 1 and 2) as 160 ng/ml worked better to give the prognosis for the clinical treatment outcome. Parameters evaluating the prognostic utility of MMP-8, when both cutoff levels were taken into account, are presented in the enclosed original article (III, Table 3). MMP-8 levels > 770 ng/ml indicated a 22%-point risk increase (from 63% pretest probability to 85% post-test probability) for the compromised (weak) treatment outcome, as the levels below 160 ng/ml indicated 25%-point risk decrease (from 63% to 38%).

In study III, the cutoff level of 4000 ng/ml differentiated high-responder sites from the low-responding-sites (pattern type 2 was named profile 2 in the original article, III) as described above. The 4000 ng/ml cutoff was also used in study I and was based on an earlier study by Mäntylä et al. (2006).

In study IV, MMP-8 response patterns could be clustered into four groups when more sites and patients were included, allowing the distinct group of high-responders to be found among the smoking patients (IV, Figure 1). Thus, the most optimal cutoff could be sought for both the baseline and the maintenance period, to differentiate between physiological and pathological levels (**Table 7**). Among the smokers the most optimal common cutoff (for all time points) was found to be 0.045 (normalized level means 4.5% from the population maximum), which corresponds to 820 ng/ml in study III. For non-smokers, the level was slightly higher, 0.085, which corresponds to 1550 ng/ml,

respectively. If all time points/visits were considered, the best cutoff level varied in range from 0.015 to 0.085 (from 270 to 1550 ng/ml) among the smokers and from 0.025 to 0.085 (from 460 to 1550 ng/ml) among the non-smokers (**Table 7**).

With the chosen 0.045 and 0.085 cutoffs, a positive test result indicated an 11%-point increase (from 49% pre-test probability to 60% post-test probability) at baseline, and the continuously detected high MMP-8 levels (the baseline and maintenance period) indicated a 39%-point increase (from 49% to 88%) for the smokers (IV). Among the non-smokers, the increase of risk was 12%-points (from 25% to 37%) at baseline and 46%-points for the continuously elevated levels (from 25% to 71%) (IV). All positive and negative likelihood ratios and post-test probabilities are presented in the original article (IV, Figure 3)

Table 7. The ROC analysis of MMP-8 levels predicting different MMP-8 response patterns (clusters) or clinical treatment outcomes at different time points of study IV. Measures in the cells present the best cutoff levels (AUC; 95% CI).

parameter		Baseline	1 month	3 months	6 months
Smokers	k = 2 clusters	0.025 (0.92; 0.87-0.97)*	0.015 (0.60; 0.46-0.74)	0.035 (0.63; 0.5-0.75)	0.075 (0.66; 0.54-0.79)*
	k = 4 clusters	0.085 (0.96; 0.92-1)*	0.015 (0.72; 0.59-0.85)*	0.065 (0.81; 0.68-0.94)*	0.065 (0.82; 0.73-0.91)*
	Clinical treatment outcome	0.055 (0.55; 0.42-0.68)	0.025 (0.67; 0.54-0.80)*	0.025 (0.73; 0.62-0.84)*	0.035 (0.67; 0.55-0.79)*
Non-smokers	k = 2 clusters	0.085 (0.97; 0.94-1)*	0.015 (0.81; 0.69-0.92)*	0.045 (0.72; 0.60-0.85)*	0.035 (0.66; 0.52-0.79)
	k = 4 clusters	0.085 (0.93; 0.88-0.99)*	0.025 (0.83; 0.73-0.94)*	0.045 (0.70; 0.57-0.82)*	0.035 (0.62; 0.49-0.76)
	Clinical treatment outcome	0.12 (0.78; 0.66-0.90)*	0.035 (0.81; 0.69-0.93)*	0.085 (0.76; 0.61-0.91)*	0.045 (0.75; 0.63-0.86)*

*statistically significant difference ($p < 0.05$).

5. DISCUSSION

5.1. Random variability and statistical properties of GCF MMP-8 levels

MMP-8 levels have expressed right-skewed distributions ubiquitously in all data sets in this thesis, as described in previous studies (Teles et al. 2009, Kinney et al. 2014, Kraft-Neumärker 2012, Mäntylä et al. 2006). Thus, levels below the mean value and extremely high “outliers” are more frequent as they were in a symmetric normal distributed data. For example, mean MMP-8 level was 1360 ng/ml for smokers at baseline and 4440 ng/ml for non-smokers and median levels were 362 ng/ml and 3620 ng/ml, respectively (III). MMP-8 data gave the best fit with the gamma distribution in the data sets among both smokers and non-smokers. There is an interesting difference in the shape parameter of gamma distributions between the smokers and non-smokers as the scale parameter is approximately the same. The shape and scale parameters describe distribution and have an analogy to mean and variance in normal (Gaussian) distributions (Nonsmokers: shape = 0.91 scale = 4.89; Smokers: shape = 0.45, scale = 4.97). A smaller shape parameter indicates a more skewed distribution. In other words, there are more sites with low GCF MMP-8 levels among the smokers, but the extremely high MMP-8 levels can be observed in both groups; smokers and non-smokers.

The skewed distribution of GCF MMP-8 levels at baseline may indicate implicitly that most sites are in steady inflammatory state of periodontitis while some sites express random bursts of activity. Low GCF MMP-8 levels are detected frequently in periodontitis sites, despite of the pocket depth or disease severity, but increasing PDs and disease severity associate with increasing mean levels of GCF MMP-8 and increasing statistical variance. In other words, higher population mean in GCF MMP-8 levels indicates a larger variance and the increased probability of MMP-8 “bursts”.

Papantonopoulos et al. (2013) studied longitudinal dynamics (5-8 years follow-up data) in disease progression of AgP and CP patients and created a model suggesting a non-linear dynamic process with “features of chaos” (statistical fractal). Both AgP and CP patients could be modeled with a similar non-linear mathematical model, in which periodontitis is described to evolve through two fixed points or attractors, namely the

stable and unstable, but the model suggested faster progression rates for AgP patients compared to CP. The model was also tested with cross sectional data (Takahashi et al. 2001), with multiple immunological parameters (from blood samples, no MMP-8 included), similarly suggesting two partially-overlapping zones (rates) of disease activity (Papantonopoulos et al. 2013).

5.2. Smoking and other modifying effects on GCF MMP-8 levels

The non-treated smoking periodontitis patients demonstrated lower average GCF MMP-8 levels compared to the non-smokers. The finding that smoking decreases oral fluid MMP-8 levels have also been observed in many other studies (Ding et al. 1994, Heikkinen et al. 2010, Ozcaka et al. 2011, Mäntylä et al. 2006). After conventional SRP treatment, MMP-8 levels decreased in both groups at first, but were inclined to run at the same levels after a few months of maintenance, except in the smokers' group of high-responders revealed with the cluster analysis.

Smoking has diverse effects on periodontal tissues. There is evidence of a dysfunctional effect on neutrophils (Palmer et al. 2005). Decreased peripheral blood flow in smokers may also have an effect on the host response, GCF volume, and biomarker content (Morozumi et al. 2004). The low baseline GCF MMP-8 levels may denote a stable, quiet period in disease activity, but in smokers it may also indicate a suppressing effect of smoking on the host response. The increase of low baseline MMP-8 levels in the type-2-response pattern sites can be explained as a recovery of physiological MMP-8 levels provoked by the treatments and a wound healing process (III and IV). In turn, very high GCF MMP-8 levels may indicate the dysfunctional hyper reactivity in neutrophil response in the smokers' high-responding sites. Whether the increase in GCF MMP-8 levels is physiological or pathological during the maintenance period depends on dynamic-feedback mechanisms, TIMP-1 levels for example, regulating enzymatic MMP-8 activity. However, the statistical risk and probability for a weak or compromised treatment outcome can be defined for different GCF MMP-8 cutoff levels and the optimal cutoff with diagnostic and prognostic properties can be determined and used in point-of-care diagnostics.

The modifying effect of smoking on the GCF MMP-8 levels was one main focus of this thesis. However, there might be also other possible host-dependent factors that can cause variability in the detected GCF MMP-8 levels not analysed in this thesis. There is some evidence that gender might have an effect on oral fluid MMP-8 levels, for example. Heikkinen et al. (2010) found that there was a significant difference in salivary MMP-8 levels between non-smoking and smoking adolescent boys and the effect was strengthened by the amount of smoking. However, the same effect was not found among same-aged girls. Actually, there was no significant difference in salivary MMP-8 levels between non-smoking boys and girls, but only among the smokers. The group of girls smoking the most (packs per year indexed) had the highest MMP-8 levels on average, but also the largest variance in the saliva MMP-8 levels. Notably, more females were included in study III relative to males. Females are also overrepresented in the groups of negative MMP-8 test results in the maintenance period (IV, Table 2). However, this result should be analysed in more detail in order to make any further conclusions. The observable effect can occur by chance or be a confounder and result from better compliance to oral hygiene instructions within the female group; this effect has been observed in many epidemiological studies (Haytac et al. 2013), for example. However, there might be some biological differences in inflammatory host responses between genders affecting also MMP-8 levels in oral fluid. Heikkinen et al. (2010) discussed, whether pubertal hormonal changes could cause differences in the salivary MMP-8 levels in the same way as was observed among pregnant females (Gürsoy et al. 2008, Gürsoy et al. 2010b, Gürsoy et al. 2010a). Nevertheless, pregnancy or other major systemic conditions or diseases having a possible effect on host response were excluded from this study. Adolescents were not included in this study either.

The positive effect of LDD on clinical parameters compared to placebo was reported in the original study of Emingil et al. (2004). The effect of LDD on the GCF MMP-8 levels was relatively small, although a significant difference was observed at 6 months compared to placebo. The effect of azithromycin on the clinical parameters or the GCF MMP-8 levels was also rather small (Emingil et al. 2012, Han et al. 2012). Nevertheless, the adjunctive medications might have had a small modifying effect on the GCF MMP-8 levels in study IV.

5.3. Comparison of MMP-8 detection methods

Measuring MMP-8 levels from oral fluids is based on immunological detection methods, which can be divided, according to the used antibody type, into assays utilizing monoclonal or polyclonal antibodies. In general, monoclonal antibodies are synthesized within a single hybridoma cell line (hybridization of myeloma cells and B cells from an immunized test animal) (Winter & Milstein 1991). The procedure leads to isolation of the hybridoma cells producing only identical antibodies detecting the same epitope on the antigen. In contrast, polyclonal antibodies originate from several B-cell lines and are capable detecting different epitopes. Quantitative assays, like all methods in this study, are based on monoclonal antibodies. Conversely, in qualitative analysis (western blot for example), polyclonal antibodies can be a better option with a better sensitivity and stronger signal, but a weaker quantitative specificity. The monoclonal antibody was used also in the western blot analysis in this work (I) to describe the affinity of the used monoclonal antibody in IFMA and other POC methods. The western blot figure (I, Figure 5) nicely demonstrates the presence of activated MMP-8 in periodontitis sites vs. the latent MMP-8 form in gingivitis sites.

Detected MMP-8 levels are quite sensitive to the antibody in the assays, as was shown in the GCF studies I and II and also in another study with oral rinse samples (Leppilahti et al. 2011). Several different cell types can express MMP-8 in inflamed tissues, as described thoroughly in the review section. MMP-8 is secreted extracellularly in the latent pro-form and activated after proteolytic cleavage (Sorsa et al. 2006). Different MMP-8 antibodies may have different affinities against the pro- and active isoforms. In addition, the MMP-8 antibodies detect molecule complexes including MMP-8 (MMP-8 bound to other molecules, heavy molecular weight complexes) or fragmented MMP-8 molecules (I, Figure 5). Neutrophil-originated MMP-8 is more glycosylated compared to *de novo* secreted MMP-8 isoforms (Hanemaaijer et al. 1997, Owen et al. 2004). There might also be diversity in the affinities of different antibodies against the glycosylated “physiological” neutrophil-derived MMP-8 if the used antibody is produced to detect the recombinant *de novo* MMP-8.

5.4. Determination of clinical treatment outcome with the cluster analysis

The major aim in periodontal research has been to determine criteria for the active disease and to predict the disease progression. Probing measures, PD and CAL itself, indicate mainly cumulative disease history, not the disease activity at the event of measurement although PD give some indication regarding the current disease. BOP and other gingival indices, denoting visually-observed inflammation in the gingiva, have moderate specificity but poor sensitivity and are poor predictors of short-term changes (Haffajee et al. 1983). However, long-standing, chronic gingivitis can be regarded as a risk factor for periodontal disease progression and tooth loss (Schätzle et al. 2003, Lang et al. 2009, Schätzle et al. 2009).

Disease progression can be detected by measuring change in PD or CAL and bone loss in radiographs. However, in the classical longitudinal follow-up study with untreated periodontitis patients, 83% of sites lacked any significant change in probing measures during the 12-months follow-up (Goodson et al. 1982). From sites with significant change, more became shallower than deeper when the patterns of disease progression and regression were analyzed. Another challenge faced in a statistical analysis of longitudinal data is that detected significant attachment loss or pocket deepening is not permanent during the follow-up. Again, in the same study with untreated patients, from the 66 (5.7% from all sites in the study) sites with a significant increase in PD during the follow-up, only 22 (1.9% from all sites in the study) retained deep levels during the follow-up as the rest of active sites had a spontaneous remission (Goodson et al. 1982). Interestingly, the deeper pockets had larger variability in both directions; spontaneous remission and progression of the lesion (Goodson et al. 1982). The same authors noticed that in a longitudinal study of 6 years, only 12% expressed attachment loss of more than 2 mm (Socransky et al. 1984). On the other hand, 40% of sites that exhibited attachment loss during the first three-year period, demonstrated no change in the next three years, and 50% of sites with no change in the first three years, had attachment loss in the last three years, respectively (Socransky et al. 1984).

In our longitudinal studies, all patients received SRP treatment and some of the patients received adjunctive medications together with SRP. If the attachment loss is a relatively rare event among untreated periodontitis patients in a one-year follow-up, it is

reasonable to assume that it should be even more unlikely among the treated patients in this study, and a significantly larger sample would be needed to detect enough “true” progressive sites for further statistical analysis. In this study, CAL levels decreased or stayed unchanged in almost all sites and only few sites presented attachment loss compared to the baseline level. Thus, a question arises, ‘what should be the outcome measure in treated periodontitis patients to compare MMP-8 or other biomarkers with, when the disease progression is a rare phenomenon to follow in a short 6-12 months study period?’

Cluster analysis of CAL change produced two distinct groups with a large and significant difference in the CAL change (III and IV). The difference in PD levels at baseline can partly explain the result, but there was a significant difference in the divergence of PD and CAL levels and the amount of GR between the groups. Interestingly, the “agnostic” cluster analysis clearly followed the dichotomy created with the traditional 2 mm cutoff to indicate the significant difference in the CAL change. However, the differences in the clinical treatment outcomes observed in this study are more likely changes in the soft tissue responses, in the amount of gingival recession, during the relatively short maintenance treatment period (6-12 months), and can be predicted, to some extent, by GCF MMP-8 levels as this study’s results suggested (III and IV). The interpretation is also supported by the other independent MMP-8 studies (Reinhardt et al. 2010, Hernandez et al. 2011). If irreversible changes in the attachment levels and the detectable bone loss in radiographs were studied, a longitudinal study with several years of follow-up would be better for the detection of the true disease progression.

5.5. Practical implications of GCF MMP-8 test

Much research has focused on GCF biomarkers to find new adjunctive diagnostic tools for periodontal diseases, and for oral fluid MMP-8 detection, the point-of-care tests have been developed and are under development (Prescher et al. 2007a, Munjal et al. 2007b, Mäntylä et al. 2003, Mäntylä et al. 2006, Sorsa et al. 2015, Heikkinen et al. 2015). The seventh European consensus workshop distinguished five different implications (susceptibility, diagnostic, prognostic, predictive, and therapeutic) for biomarkers to be used in periodontal diagnostics described in the review section (Kinane et al. 2011). This

thesis focused on three of these five implications; diagnostic, prognostic and therapeutic utility of GCF MMP-8 at the site level.

Making a general diagnosis of periodontitis, detecting the periodontal attachment loss, and differentiating periodontitis from gingivitis or healthy sites and patients, should be easily done by a dental professional. However, there are situations when dentists are not available and resources are too low to carry out a periodontological examination for the whole population in question. Thus, there is a need for a reliable but cheap and simple way to perform a non-invasive test for screening and diagnostic purposes (Nwhator et al. 2014, Sorsa et al. 2015, Heikkinen et al. 2015).

Oral fluid MMP-8 tests, employing the antibody originally described by Hanemaaijer et al. (1997), and later by Mäntylä et al. (2003), Leppilahti et al. (2011), Nwhator et al. (2014), and Heikkinen et al. (2015), have shown quite good agreement with the periodontal status. The first MMP-8 POC test with the antibody used in this study was a qualitative dip-stick test with good agreement with the IFMA analysis ($\kappa = 0.81$) and had the sensitivity of 0.64 and the specificity of 0.92 to differentiate periodontitis sites from gingivitis or healthy sites (Mäntylä et al. 2003). The portable quantitative POC device (dentoAnalyzer®) based on the technology developed originally for the detection of *Streptococcus sobrinus* (Munjal et al. 2007b, Munjal et al. 2007a) and the dip-stick test was compared with IFMA again with good correlation ($r = 0.95$, I). Nwhator et al. (2014) found that the commercially available lateral-flow (PerioMarker®) MMP-8 test had very high sensitivity to detect signs of chronic periodontitis (95%), poor oral hygiene (96%), and BOP (83%) from oral rinse samples, although the specificity of the test was quite low. In a very recent pilot study, initial signs of periodontitis could be detected with very few false positives (sensitivity 48- 76.5%; specificity 96.7-100%) in a population of adolescents by a commercial chair-side test (PerioSafe®) based on the same monoclonal antibody as in the PerioMarker®, the test-stick, or IFMA (Heikkinen et al. 2015). Such a test is eligible to screen people for periodontitis cost efficiently, in circumstances with low dental care resources (Nwhator et al. 2014, Heikkinen et al. 2015). MMP-8 tests from GCF samples in site-level study II, and also by Mäntylä et al. (2003), showed a good diagnostic performance comparable to the mouth-rinse study of Heikkinen et al. (2015).

It is also pertinent to question whether there are differences in the GCF MMP-8 levels between healthy or gingivitis sites in non-periodontitis patients and so called

'shallow' healthy or gingivitis sites ($PD < 4$ mm) in periodontitis patients. Few studies address the question. Figueredo et al. (2004) found significant decrease in the GCF MMP-8 levels after treatment in both shallow (with gingivitis) and deep pockets of periodontitis patients, but periodontitis sites had higher GCF MMP-8 levels after treatment compared to the shallow ones. In a cross-sectional study, the same authors compared GCF MMP-8 levels between gingivitis sites of non-periodontitis patients, gingivitis sites (shallow pockets) of periodontitis patients, and real periodontitis sites (with deep pockets) and found no difference in the GCF MMP-8 levels between the groups (Figueredo et al. 2005). Passoja et al. (2008) have an interesting aspect in their study by analysing the GCF MMP-8 levels in the shallow ($PD < 4$ mm) crevices of periodontitis patients. They found that GCF MMP-8 levels were typically lower in the group of shallow crevices compared to diseased sites ($PD \geq 4$ mm). However, they found that GCF MMP-8 levels in shallow crevices associated with the extent of attachment loss in periodontitis patients. All of these referred studies applied different MMP-8 detection methods, with different antibodies, and diverse GCF sampling methods (Figueredo et al. 2004, 2005) relative to the methods employed in this study and thus the results are not directly comparable. Nevertheless, it was noticed earlier with IFMA method that the GCF MMP-8 levels were at increased levels even in stable periodontitis sites after successful treatment compared to GCF MMP-8 levels in healthy sites (Mäntylä et al. 2006, Sorsa et al. 2015). In this thesis, the data inclusion criteria for a periodontitis site was $PD \geq 4$ mm (III) and $PD \geq 5$ mm (IV), and the so-called 'shallow sites' of periodontitis patients were excluded from the analysis.

Predicting disease progression and giving a prognosis for treatment is a more challenging task in comparison to a simpler screening or a preliminary diagnosis of periodontitis. The difficulty in finding the *prognostic* or *predictive* biomarker or a composition of markers in periodontitis is most likely based on the nature of the disease itself. The disease itself progresses in periods of exacerbations and remissions (Socransky et al. 1984b, Schätzle et al. 2009, Mdala et al. 2014). An interesting finding is that most of the oral fluid, inflammatory host-response-reflecting biomarkers, such as MMP-8, -14, azurocidin, and MPO (II), obey skewed, non-normal (non-Gaussian), distributions and implicitly denote inflammation as a burst-like phenomenon. On the other hand, changes in clinical parameters seem to be more near the assumption of normality (Gaussian distribution). Longitudinal dynamics and a cumulative effect of

inflammatory bursts on the clinically-observable disease progression is a topic for further detailed study for better understanding.

Low GCF MMP-8 levels at periodontitis sites are relatively frequent, but during the burst of activity, MMP-8 levels (and other inflammatory biomarkers) can multiply in an exponential fashion (II, III, IV). Thus, no linear nor deterministic association is evident between inflammatory biomarkers and the changes in clinical signs of periodontitis. The cutoff between physiological and pathological expression is probably not directly obvious in biomarker levels, but rather in the nonlinear dynamics of inflammatory cascades and the balance between burst and quiet periods (Papantonopoulos et al. 2013, Papantonopoulos et al. 2014). The detection of a single biomarker from the complex network of abundant interacting molecules might be considered to be a naïve approach. However, the cutoff between extraordinary high “signals” and frequently observed “noisy” fluctuations of the marker can be sorted out by exploring different longitudinal response patterns. It can be compared if the cutoff can differentiate also between pathological and physiological behavior, and further estimate the performance of the biomarker test, for example with likelihood ratios. A positive likelihood ratio of 1 corresponds to “a coin flipping” as the ratio of 5 is considered a rule of thumb cutoff for good performance of the biological/biomarker test (Ray et al. 2010). The single GCF MMP-8 measurement at baseline (study IV: smokers 1.54 and non-smokers 1.88; study III: Smokers 3.3) could not achieve the level of 5. Higher +LR in study III can probably be explained by a more homogenic population compared to the pooled data in study IV. MMP-8 tests during the maintenance period achieved a better prognostic utility. Continuously high MMP-8 levels were apparent during the maintenance period (twice as positive result), reaching a +LR around 5 in both the smokers’ (5.0) and non-smokers’ groups (4.22). If the baseline test and the maintenance test was combined, the double-positive result (+baseline and + maintenance period) indicated +LR of 8.23 among the smokers and 4.12 among the non-smokers. In contrast, the double negative result yield a -LR of 0.55 in smokers and 0.48 in non-smokers. From this data it can concluded that the GCF MMP-8 test has a moderate prognostic utility on the SRP treatment and a good performance to monitor the treatment outcome quantitatively in both groups of smokers and nonsmokers when the whole 6-months follow-up from baseline to the end of maintenance period is considered.

It can be speculated that the GCF MMP-8 levels are able to reflect biological differences caused by smoking on inflammatory host response: neutrophil granulocyte functions for example. Actually, MMP-8 could give a prognosis for the site-level clinical treatment outcome more specifically among the smokers in studies III and IV. High-responder results that clustered separately in study IV, support this hypothesis.

The single “most optimal” cutoff level was chosen to study the performance of a simple MMP-8 test. However, it seems that the best cutoff (estimated from the population) is different at baseline and during maintenance period as seen from the range of optimal cutoffs (for the smokers 0.015-0.065 and for the non-smokers 0.025-0.085) conducted from the ROC analysis. Among the non-smokers at baseline, the best cutoff (0.085 nonsmoker) is higher compared to the maintenance period (0.025-0.045) as among smokers the best cutoff was at baseline 0.055, decreased after SRP treatments but tended to increase again during the maintenance period (0.065 at 6 months). Among the non-smokers, the result is logic. Biofilm and calculus have provoked inflammation reflected by the increased GCF MMP-8 levels at untreated sites and by the decreased levels after the treatment. However, the higher “optimal” cutoff level among smoking patients compared to non-smokers during the maintenance period can be explained with the group of high-responders that was clustered as an own group (**Table 7**).

Improving host-response modifying treatments is another reason that can support biomarker analysis in addition to focus of improving periodontal examination and the treatment. The effect of LDD to improve treatment outcome after conventional SRP has been shown in many independent studies and the Food and Drug Administration (of U.S.) has approved LDD as an adjunct medication to the conventional SRP for the treatment of chronic periodontitis (Caton & Ryan 2011). The effect of LDD is based on by its ability to chelate Zn^{2+} ion, a cofactor of MMPs, and inhibit enzymatic activity. However, there are some studies where non-smokers benefit more from LDD when compared with smokers (Preshaw et al. 2005) and one study with smoking patients where no significant difference was observed in the treatment outcome between LDD and the placebo (Needleman et al. 2007). An interesting study hypothesis would be to study the effect of LDD during the maintenance period based on different MMP-8 levels at baseline. If sites/patients already had low MMP-8 levels at baseline, would they have a different treatment outcomes with the adjunctive LDD or the placebo compared to sites/patients with high MMP-8 levels at baseline? Although, one data from the clinical trial with LDD

was presented here (IV), the number of sites with LDD treatment was too small to study this aspect.

MMP-detecting antibodies/assays do not necessarily differentiate between LDD inhibited and active MMPs, however it is shown in many clinical studies that MMP-8 levels decrease slightly more in LDD groups compared to the placebo (Kormi et al. 2014, Emingil et al. 2004, Reinhardt et al. 2010), suggesting a secondary effect of LDD on the MMP-8 expression. The effect was very clear in serum samples of coronary bypass patients who received conventional antibiotic dose of doxycycline 100 mg/day for 4 months (LDD dose is 20 mg/day) to eradicate *C.Pneumoniae* to prevent secondary cardiovascular events (Kormi et al. 2014).

6. CONCLUSIONS

There is significant variability between quantitative, immunological detection methods, based on different monoclonal antibodies, to determine GCF MMP-8 levels. Methods based on the same monoclonal antibody should be employed if oral fluid MMP-8 levels are compared between independent studies.

Smoking has a various effect on GCF MMP-8 levels and MMP-8 response patterns after SRP treatment. Smokers have decreased mean GCF MMP-8 levels, which should be taken into account in a statistical analysis and when the diagnostic cutoff levels for MMP-8 are defined. There is also a group of smoking patients with very high GCF MMP-8 levels, especially during the maintenance period, i.e. they respond to treatments with the exceptionally high MMP-8 levels. These high-responders have increased risk for compromised treatment outcomes and could potentially be targets for an adjunctive medication and treatments.

Periodontitis sites can be identified diagnostically from gingivitis or healthy sites by utilizing GCF MMP-8 levels. GCF MMP-8 levels have also a moderate prognostic utility to predict site-level periodontal treatment outcome (periodontal soft tissue changes) and a good utility to quantitatively reflect therapeutic response on the given treatment.

7. ACKNOWLEDGEMENTS

This thesis was carried out at the Department of Oral and Maxillofacial Diseases (former Institute of Dentistry), Biomedicum Helsinki, Faculty of Medicine, University of Helsinki during the years 2009- 2016. The research project was financially supported by the Etelä-Pohjalaisen Osakunan stipendisäätiö Foundation, The Finnish Dental Society Apollonia, the Finnish Doctoral Programme in Oral Science and Academy of Finland.

I wish to thank all those who have contributed this study:

Professor Jarkko Hietanen and docent Hanna Thóren the former Deans of the Institute of Dentistry, Christian Lindqvist, the present Head of Department of Oral and Maxillofacial Diseases and Kirsti Kari, MSc, the former head of the scientific laboratory of the Institute of Dentistry are acknowledged for providing excellent research facilities.

I wish to express profound gratitude to my supervisor Docent Päivi Mäntylä. Your strong competence in making research and wide understanding in the field of periodontology but also warm- hearted encouraging have gave me privileged guidance to make this thesis to conclusion.

I also want to thank most sincerely my supervisor professor Timo Sorsa. Your innovations and long experience in making science have been the foundation for this work. Nevertheless, your open-minded and optimistic attitude have also given me strong support and encouraged to explore new paths in the research.

I would like to thank warmly Professor Andrea Mombelli and Associate Professor Marja Laine for their precious time and valuable and constructive comments in revising this thesis. I wish to express my gratitude to Professor Rodrigo López for accepting the invitation of being the official opponent in the dissertation.

Professor Veli-Jukka Uitto and Docent Kimmo Suomalainen to give their guidance in the thesis committee.

I am very grateful to all the collaborators working with this project:

Taina Tervahartiala for encouraging and guidance in laboratory works.

Gülnur Emingil and Buket Han in Ege University, İzmir, Turkey. Marcela Hernández, Romina Brignardello-Petersen, Jorge Gamonal and Patricia Hernández- Ríos in the University of Chile, Santiago, Chile.

Aleksi Kallio, giving his priceless expertise in statistics but also for the friendship and discussions having its roots in the House of Ostrobotnia.

I wish to thank all my co-workers, both past and present, at the Institute of Dentistry and Doctoral School in Oral Sciences.

I also wish to thank all my colleagues and staff members in the dental clinics in Siikalatva and Haapavesi. I am especially grateful to my superior Tuulikki Ulander who has allowed me to combine scientific and clinical work in a flexible way.

I want to thank my parents Kaisa and Mikael Leppilahti for always believing in my skills and encouraging me to go forward, and sisters Maria, Liisa and Laura for your unconditional support.

Finally I want to thank lovingly my wife Mari-Erika and children Touko and Vappu all the support and understanding during these years.

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