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DISSERTATION

Determination of active Matrix-Metalloproteinase 8 (aMMP-8) levels in the Gingival Crevicular Fluid as a Diagnostic Test during Periodontal Maintenance Therapy

zur Erlangung des akademischen Grades
Doctor medicinae dentariae (Dr. med. dent.)

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Table of Contents

List of abbreviations

aMMP-8	active matrix metalloproteinase 8
AgP	aggressive periodontitis
API	approximal plaque Index
AUC	area under the curve
BOP	bleeding on probing
CRF	case report form
ChP	chronic periodontitis
CAL	clinical attachment level
CI	confidence interval
ELISA	enzyme-linked immunosorbent assay
et al.	*et alii - and others*
ECM	extracellular matrix
Fig.	figure
GCF	gingival crevicular fluid
IL	interleukin
IQ	interquartile distance
LPS	lipopolysaccharides
LDD	low-dose doxycycline
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase
µg	microgram
µL	microlitre
mL	millilitre
mm	millimetre
ng	nanogram
OR	odds ratio
OPG	osteoprotegerin
PBI	papilla bleeding index
ppm	parts per million
%	percent
PISF	peri-implant sulcus fluid
pg	picogram

PI	plaque index
PD	pocket depth
POC	point-of-care
PMN	polymorphonuclear leukocyte
P. gingivalis	*Porphyromonas gingivalis*
PGE_2	prostaglandin E_2
ROC curves	receiver operating characteristic (ROC) curves
RANK	receptor activator of nuclear factor-kappa beta
RANKL	receptor activator of nuclear factor-kappa beta ligand
Rec	recession
RAL	relative attachment level
SRP	scaling/root planing
SBA	soluble biotinylated collagen assay
SDD	sub-antimicrobial dose doxycycline
SPT	supportive periodontal therapy
Th	T helper cell
IFMA	time-resolved immunofluorometric assay
TIMP	tissue inhibitors of metalloproteinases
T. denticola	*Treponema denticola*
TNFα	tumor necrosis factor α
V	visit

Abstract

Objectives: Clinical parameters used for the monitoring of periodontal therapy focus on past episodes of tissue destruction and are of little prognostic value. The enzyme, active matrix metalloproteinase 8 (aMMP-8), is a mediator of tissue destruction in periodontal inflammation. In cross-sectional studies, gingival crevicular fluid (GCF) levels of aMMP-8 differentiated healthy/gingivitis sites from periodontitis sites. The aim of this study was to determine if GCF aMMP-8 levels correlate with clinical periodontal parameters and if they predict disease progression (relapse) during supportive periodontal therapy (SPT).

Methods: 34 periodontitis patients were treated with scaling/root planing (SRP) with or without systemic antibiotics. Then three subsequent SPT visits followed at intervals of three months including oral hygiene instructions and clinical measurements and SRP at the last two visits. Probing pocket depths (PD) and recessions (Rec) were measured using an electronic constant-force periodontal probe, and clinical attachment levels (CAL) were calculated. Bleeding on probing (BOP) was registered as present or absent at four sites/tooth. Four GCF aMMP-8 test strip samples/patient were obtained at four tooth sites with initial PD≥4mm before the treatment, then after two weeks, and at two consecutive SPT visits. The concentration of aMMP-8 was quantified in the external laboratory (Dentognostics GmbH, Jena) using an enzyme-linked immunosorbent assay (ELISA) which detects mainly the active form of the enzyme with specific antibodies. Different definitions of patient-based disease progression were used in evaluating changes between two consecutive visits: increase of percentage of sites with PD≥5mm/patient; increase of full-mouth PD; increase of full-mouth CAL; increase of PD in sample sites; ≥0.5mm increase of PD in sample sites; increase of CAL in sample sites; ≥0.5mm increase of CAL in sample sites. The ability of pooled aMMP-8 levels to predict patient-based relapse was tested by the construction of receiver operating characteristic (ROC) curves. **Results**: The levels of aMMP-8 correlated with PD at the initial visit and with BOP at initial and first maintenance visit. Periodontal treatment resulted in the reduction of GCF aMMP-8 levels. Regardless of the definition of disease progression, aMMP-8 levels did not predict disease progression found at the subsequent visit ($p>0.05$). **Conclusion**: aMMP-8 levels in GCF correlate inconsistently with the clinical parameters of periodontitis. The levels of aMMP-8 sampled from a limited number of GCF sites do not predict the progression of periodontitis during SPT in a group of patients with chronic or aggressive periodontitis.

Key words: MMP-8, periodontitis, progression

Zusammenfassung

Ziele: Klinische Parameter zur Bewertung der Parodontaltherapie konzentrieren sich auf vergangene Episoden der Gewebezerstörung und sind von geringer prognostischer Aussagekraft. Das Enzym aktive Matrix-Metalloproteinase 8 (aMMP-8) ist ein Mediator des Gewebeabbaus bei parodontaler Entzündung. In Querschnittsstudien unterschieden sich gesunde/Gingivitis Stellen von parodontal betroffenen Stellen in aMMP-8-Levels in der Sulkusflüssigkeit (GCF). Das Ziel der Studie war die Prüfung, ob die aMMP-8-Levels in der GCF mit klinischen parodontalen Parametern korrelieren und ob sie die Progression der Krankheit (Rezidiv) während der unterstützenden Parodontitis-Therapie (SPT) vorhersagen. **Methode**: 34 Patienten mit Parodontitis wurden mit Scaling/Root planing (SRP) mit oder ohne systemische Antibiose behandelt. Es folgten drei SPT-Sitzungen im Abstand von drei Monaten inklusive Mundhygieneinstruktionen und klinischen Messungen sowie zusätzlichem SRP während der letzten zwei Sitzungen. Sondierungstiefen (PD) und Rezessionen (Rec) wurden mithilfe elektronischer, druckkalibrierter Parodontalsonde gemessen, und die klinischen Attachmentlevels (CAL) wurden berechnet. Blutung auf Sondierung (BOP) wurde als vorhanden oder nicht vorhanden an vier Seiten/Zahn registriert. Vier GCF aMMP-8 Proben/Patient wurden mit Teststreifen an vier Stellen mit initialen PD≥4mm vor der Behandlung, nach zwei Wochen und bei zwei aufeinander folgenden SPT-Sitzungen gewonnen. Die Konzentration der aMMP-8 wurde im externen Labor (Dentognostics GmbH, Jena) mithilfe eines Enzyme Linked Immunosorbent Assays (ELISA) gemessen, das hauptsächlich die aktive Form des Enzyms mit spezifischen Antikörpern detektiert. Unterschiedliche Definitionen der patientenbezogenen Progression der Krankheit wurden verwendet, welche die Unterschiede zwischen zwei aufeinanderfolgenden Sitzungen beschrieben: Anstieg des Prozentsatzes der Stellen mit PD≥5mm/Patient; Anstieg der Full-Mouth-PD; Anstieg des Full-Mouth-CAL; Anstieg der PD an den Entnahmestellen; ≥0.5mm Anstieg der PD an den Entnahmestellen; Anstieg von CAL an den Entnahmestellen; ≥0.5mm Anstieg von CAL an den Entnahmestellen. Die diagnostische Validität der gepoolten aMMP-8-Messung zur Prognose eines patientenbezogenen Rezidivs wurde durch die Berechnung von Grenzwertoptimierungskurven (ROC-Kurven) getestet. **Ergebnisse**: Die aMMP-8-Levels korrelierten mit PD während der initialen Sitzung sowie mit BOP während der initialen Sitzung und beim erstem Recalltermin. Die parodontale Behandlung führte zur Reduktion der GCF aMMP-8 Konzentrationen. Unabhängig von der Definition der Progression sagten die aMMP-8-Levels eine Progression der Parodontitis bis zur folgenden Sitzung nicht vor ($p > 0,05$). **Schlussfolgerung**: Die aMMP-8-Levels in GCF korrelieren inkonsistent mit den klinischen Parametern der Parodontitis. Die Bestimmung der aus einer begrenzten Anzahl von GCF-Entnahmestellen gewonnen aMMP-8 sagen die Parodontitisprogression während SPT in einer Gruppe von Patienten mit chronischer oder aggressiver Parodontitis nicht vor.

Schlagwörter: MMP-8, Parodontitis, Progression

1. Scientific background

1.1 Introduction

Periodontal diseases and conditions comprise situations in which the pathological process affects either only the soft tissue surrounding the teeth (gingiva) or also the deeper, tooth-supporting hard and soft tissue structures (periodontium) (Kinane, 2001, Armitage, 1999). Apical periodontitis is an inflammatory condition around the apex of a tooth root caused by bacterial invasion originating from the pulp of the tooth, whereas marginal periodontitis, bacteria-induced inflammatory process of coronal part of periodontium, is clearly the most significant of those conditions as it constitutes the main cause of permanent tooth loss in some populations, outnumbering dental caries (Glockmann, 2011). Furthermore, periodontal inflammation is linked to systemic diseases such as cardiovascular disease, adverse pregnancy outcomes, stroke or diabetes (Ramseier *et al*., 2009, Mealey and Oates, 2006, Agueda *et al*., 2008).

Periodontitis is a multifactorial disease in which pathogens are necessary as an etiological factor, however not sufficient for the disease to occur. Bacteria living in and forming the biofilm in the oral cavity may account for only 20% risk of periodontal disease; however other factors, such as genetic, environmental ones and, finally, the host response must be present for the disease to occur (Kinane and Mark Bartold, 2007, Lang and Tonetti, 2003, Michalowicz, 1994). Paradoxically, it is the host's inflammatory response in defence against the microbial challenge which finally leads to the destruction of host's own periodontal tissue and, subsequently, to tooth loss (Page and Kornman, 1997, Giannobile *et al*., 2009). Clinically, the stability of periodontal health depends on a dynamic equilibrium between the bacterial challenge and an effective host response.

Patients with a history of periodontal disease after undergoing initial periodontal therapy are recognised as a moderate to high-risk group for recurrent periodontal infection. Hence, in contrast to the population without history of periodontitis, they should participate in a life-long recall programme comprising maintenance periodontal therapy (also called supportive periodontal therapy) which consists usually of 2 - 4 appointments per year (Lang and Tonetti, 2003). Current diagnostic methods are still based mainly on the clinical parameters introduced more than 50 years ago, like probing pocket depths, bleeding on probing, clinical attachment level, tooth mobility and alveolar bone level assessed on the radiographs (Kinney *et al*., 2007, Armitage, 2004b, Armitage, 2013).

Based on these clinical findings it is assumed that pathologically deepened periodontal pockets as well as bleeding on probing are signs of unstable periodontium. Therefore, a subgingival debridement (cleaning of the root surface) should be performed again to prevent tissue loss and disease progression (Renvert and Persson, 2004). However, it has been proven that these standard clinical parameters are of only minor prognostic value and they may lead to undertreatment of some patients (tooth loss or general health complications due to delayed treatment) or overtreatment of other patients (recessions, tooth hypersensitivity, root caries risk, as a result of frequently repeated mechanical treatment) (Reiker *et al*., 1999, Sykes, 2007, Renvert and Persson, 2002). What is more important, we are able to determine clinically and radiologically only ongoing inflammation processes or already irreversible tissue loss (Kraft-Neumarker *et al*., 2012). Therefore, there is a need for innovative diagnostic tests and identification of biomarkers reflecting host response to bacterial challenge which precedes clinical signs of inflammation in the periodontal tissues, marking the period prior to those visible changes (Fine *et al*., 2009). Optimally, these diagnostic indicators should determine the current activity of the disease, predict sites prone to future breakdown and assess tissue response to the treatment (Giannobile *et al*., 2009).

Matrix metalloproteinases (MMP), a group of host cell-derived calcium-dependent, zinc-containing endopeptidases participate in the normal turnover of periodontal tissues and they are also responsible for the degradation of most extracellular matrix proteins during periodontal disease (Uitto *et al*., 2003, Sorsa *et al*., 2004). Collagenase-2, or matrix metalloproteinase-8 (MMP-8), the most prevalent MMP found in diseased periodontal tissue, appears to be a clinically useful point-of-care biomarker for periodontal and peri-implant diseases detectable in oral fluid such as gingival crevicular fluid (GCF), peri-implant sulcus fluid (PISF), mouth rinse and saliva (Sorsa et al., 2011, Leppilahti et al., 2015, Wohlfahrt et al., 2014). Increased amount and activity of MMP-8 correlate with the severity of periodontal disease (Lee *et al*., 1995, Kinane *et al*., 2003, Leppilahti *et al*., 2014a, Gursoy *et al*., 2013). Subgingival debridement has proven to decrease the level and activity of MMP-8, whereas pockets with poor response to treatment tend to have a persistently elevated or fluctuating level of this enzyme (Mäntylä et al., 2006, Konopka et al., 2012, Kinane et al., 2003). MMP-8, according to some data, can be used to differentiate periodontitis from gingivitis and healthy sites or periodontally affected patients from the healthy ones, as well as to monitor the treatment of periodontitis (Mäntylä et al.,2003, Prescher et al.,2007, Ramseier et al.,2009, Leppilahti et al., 2015).

At present, there are several different commercial MMP-8 tests available on the market, however they are more suitable for the confirmation of clinical findings on the biochemical level, rather than having a prognostic value. There is a lack of sufficient longitudinal studies, evidence-based information and protocols, which could offer the clinician additional data helping him in the decision-taking process regarding the treatment modalities during the maintenance phase of periodontal therapy and to clarify the prognosis. The main goal of our study was to determine if aMMP-8 levels in GCF measured at one time point can predict periodontitis progression between the given time point and the subsequent visit and thus enable the dentist to take preventive action before an irreversible destruction occurs.

1.2 Classification and prevalence of periodontal diseases and conditions

According to the definition of American Academy of Periodontology, the periodontal diseases comprise the pathologic processes affecting the periodontium, i.e. the tissues that invest and support the teeth including the gingiva, alveolar mucosa, cementum, periodontal ligament, and alveolar supporting bone (American Academy of Periodontology, 2012a). The currently valid classification system, presented in Table I, originates from 1999, and was developed during the International Workshop for a Classification of Periodontal Diseases and Conditions in the USA, and additionally interpreted in 2014 by the American Academy of Periodontology Board of Trustees (Armitage, 1999, American Academy of Periodontology, 2015). The classification was adopted by the German Society of Periodontology in 2002 (Deutsche Gesellschaft für Parodontologie, 2002).

Table I: Classification of periodontal diseases and conditions

I	Gingival Diseases	
II	Chronic Periodontitis	A. Localized
		B. Generalized
III	Aggressive Periodontitis	A. Localized
		B. Generalized
IV	Periodontitis as a Manifestation of Systemic Diseases	
V	Necrotizing Periodontal Diseases	
VI	Abscesses of the Periodontium	
VII	Periodontitis Associated with Endodontic Lesions	
VIII	Developmental or Acquired Deformities and Conditions	

Periodontitis, bacteria-induced inflammatory process of periodontium, which leads to the destruction of the tooth-supporting structures including periodontal ligaments, bone and soft tissues, is clearly the most significant of these conditions as it constitutes the main cause of permanent tooth loss in some populations (Kinane, 2001). In a recent study concerning the main causes of tooth loss in German population, periodontitis was found to be the leading cause, followed by caries, in patients over 40 year old (Glockmann, 2011). An average inflamed periodontal tissue in a diseased patient extends over the area of 8 – 20 cm^2 or even more in severe cases, which corresponds to an open wound of the size of the human palm (Slots, 2003). Periodontal inflammation is also linked to systemic diseases, increasing the risk of developing a cardiovascular disease almost two times, premature birth by two to four times and diabetes mellitus type 2 up to two times (Azarpazhooh and Tenenbaum, 2012, Agueda *et al*., 2008, Demmer *et al*., 2008). Considering the above hazards, a meaningful step was taken by the German Dental Association (BZÄK), who adopted in 2004 the goals for oral health following World Health Organisation's (WHO) recommendations designed to decrease the prevalence of periodontitis to 10% in the age group of 35 - 44 and to 20% in that of 65 - 74 by the year 2020. However, data from the fourth German Oral Health Study (DMS IV) show the opposite tendency, with periodontitis prevalence increasing from 46.13% in 1997 to 73.2% in 2005 in the middle aged patients group (Schiffner *et al*., 2009).

1.3 Aetiology and pathogenesis of periodontitis

Oral bacteria colonise exposed tooth surfaces rapidly after oral hygiene procedures are performed or during tooth eruption. Within a few minutes, denuded areas are covered with the acquired pellicle, consisting primarily of salivary proteins and glycoproteins, as well as extracellular molecules of bacterial origin (Marsh and Bradshaw, 1995). These adsorbed molecules function as adhesins or receptors for selected primary bacterial colonizers, which include facultative anaerobic Gram-positive cocci and rods - *Streptococci* and *Actinomyces* species that can be observed within the first 2-4 hours (Nyvad and Kilian, 1987). They coaggregate with *Fusobacterium nucleatum*, which seems to play a bridging role for late colonizing bacteria, mostly Gram-negative ones such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*, and *Treponema denticola* (Kolenbrander and London, 1993). Three species, *P. gingivalis, Tannerella forsythia and T. denticola*, called the "red complex", were

assumed as putative bacterial pathogens, associated with the progression of chronic periodontitis (Socransky *et al*., 1998).

Microorganisms from dental plaque form biofilms which are defined as bacterial communities embedded in a matrix and adhering to each other and/or to surfaces or interfaces (Costerton *et al*., 1995). Biofilm undergoes maturation and gradually creates unique spatial structuring; within few days a shift of plaque composition and structure occurs due to lowering of the oxygen concentration and change of the redox potential. Bacterial cells multiply, which leads to an increase in plaque volume and the production of extracellular polymers which form a biofilm matrix (Allison, 2003). The matrix functions as a scaffold for the embedded bacteria, and provides integrity and resistance to environmental conditions (Marsh *et al*., 2011). It retains water and nutrients and can prevent penetration of certain molecules such as antimicrobial agents, e.g. chlorhexidine (Zaura-Arite *et al*., 2001). Bacteria can communicate with each other by exchanging genetic information and through quorum sensing, which enables coordination of the gene expression depending on the population density (Suntharalingam and Cvitkovitch, 2005). The gingival sulcus, and especially the col area, region between two papillae in the interdental region, create protected niches favouring biofilm formation and retention (Dentino *et al*., 2013).

Periodontitis is a multifactorial, inflammatory disease in which pathogens are necessary as an etiological factor, but not sufficient for the disease to occur (Offenbacher, 1996). Bacterial biofilm in the oral cavity accounts for only 20% risk of periodontal disease, however, other factors, such as genetic, environmental ones and, finally, the susceptible host response must be present for the disease to occur and progress (Kinane and Mark Bartold, 2007, Lang and Tonetti, 2003, Michalowicz, 1994). Paradoxically, it is the host's inflammatory response as a defence to the microbial challenge which finally leads to the destruction of host's own periodontal tissue and subsequent tooth loss (Page and Kornman, 1997, Giannobile *et al*., 2009) (see Figure 1).

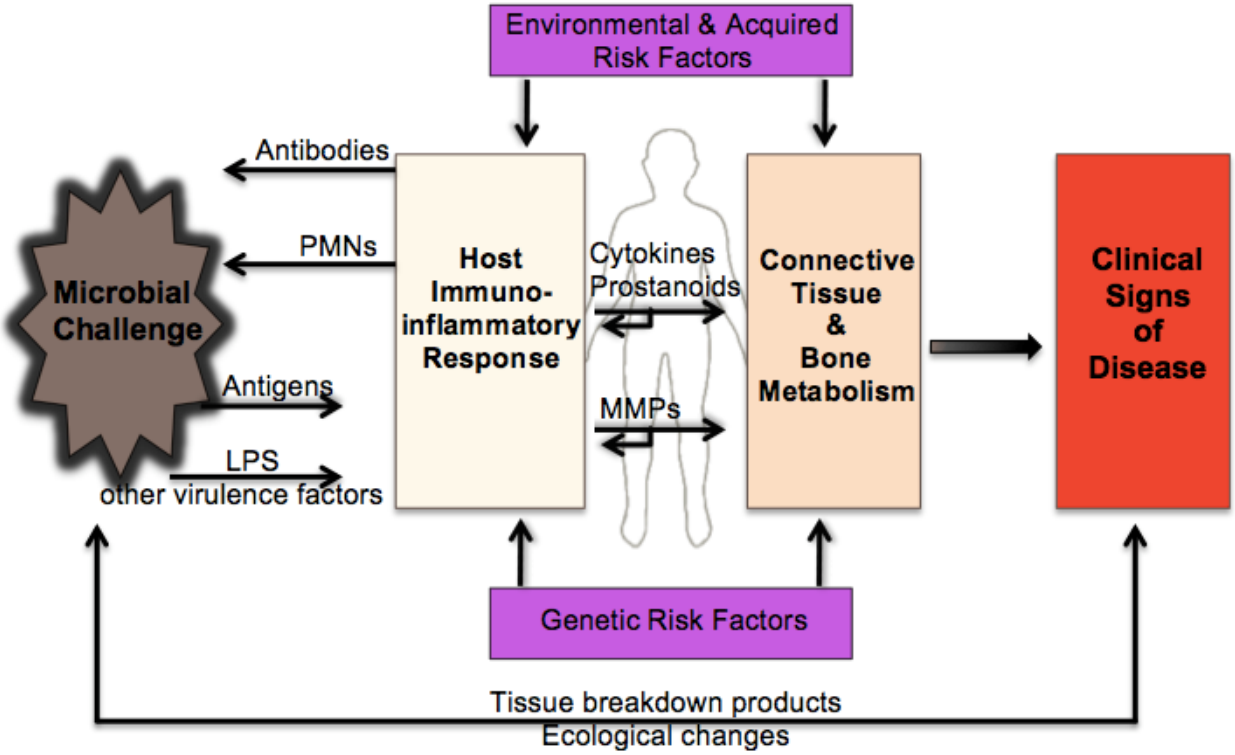

Fig. 1 Pathogenesis of periodontitis; bacterial pathogens, susceptible host and risk factors such as environmental, acquired or genetic factors have to be present for the development of the disease. Figure modified after (Page and Kornman, 1997, Kornman, 1999).

Clinically, the stability of periodontal health depends on a dynamic equilibrium between bacterial challenge and effective host response. In a healthy person, biofilm and host defence system exist in mutually beneficial symbiosis (Marsh and Percival, 2006). When the dynamic balance of microbial homoeostasis is broken, an ecological shift, a reorganisation of structure and composition of biofilm occurs (Socransky *et al*., 1998). Subjects develop clinical signs of gingivitis such as bleeding, redness and oedema, which are fully reversible after the removal of etiological factors (Loe *et al*., 1965). In susceptible patients, the disrupted homoeostasis can lead to alterations in bone and connective tissue metabolism (Kornman, 2008). Clinical signs of tissue inflammation and loss of attachment follow until a fragile balance is reached where destruction is brought to a standstill and limited tissue repair can take place, followed by another episode of disease activity (Page *et al*., 1997). Irreversible damage to tooth supporting structures, the periodontium, which can lead finally to tooth loss, can be clinically diagnosed as periodontitis (Pihlstrom *et al*., 2005).

Bacterial components and bacterial waste products, such as lipopolysaccharides (LPS-endotoxins), fatty acids, proteases and metabolic toxins are present in the biofilm covering the tooth surfaces and gingival margins (Kornman *et al*., 1997). In a healthy

person, most of the bacteria released continually from the biofilm and their products are flushed away by saliva and crevicular fluid flow before they manage to evoke significant host response (Schroeder and Listgarten, 1997). What is more, a host immune and inflammatory response may be triggered, working usually in a protective way, but both host hypo-responsiveness and hyper-responsiveness to microbial challenge can result in increased tissue destruction (Preshaw *et al*., 2004b).

Soft tissue degradation and bone resorption occur by the activation of several concomitant pathways (see Figure 2). On one hand, bacterial antigens such as LPS, which diffuse through junctional epithelium into the gingival connective tissue, can stimulate the mastocytes to the production of vasoactive amines and TNFα and consequently lead to increased vascular permeability. This facilitates the recruitment of inflammatory cells (PMNs, macrophages) to the tissue (Ohlrich *et al*., 2009).

Furthermore, bacterial antigens and bacterial products like peptidoglycans are recognized by toll-like receptors on the host cell surface and can initiate an inflammatory response by releasing cytokines (TNFα, IL-1β, IL-6, IL-8), PGE_2 and finally matrix metalloproteinases (MMPs) from host cells, including periodontal ligament fibroblasts, causing gingival connective tissue degradation (Mahanonda and Pichyangkul, 2007, Nishikawa *et al*., 2002).

Antigen-presenting cells such as macrophages or B cells, once in contact with oral pathogens, interact with T helper cells (Th0) that differentiate to various subsets, such as Th1, Th2, Th17 and regulatory Tregs, which produce further inflammatory mediators. Those mediators, including TNFα, PGE_2, IL-6 and IL-1β, activate indirectly the differentiation of osteoclasts precursors into osteoclasts by upregulating the receptor activator of nuclear factor-kappa beta ligand (RANKL) expression and inhibiting osteoprotegerin (OPG) expression in host cells, such as osteoblasts. Osteoclast precursor cells differentiate through the receptor activator of nuclear factor-kappa beta (RANK) into osteoclasts, which are capable of alveolar bone resorption (Boyce and Xing, 2007). The normal balance between bone formation and bone resorption is shifted towards catabolic processes, and soft and hard tissue degradation can be found during a patient's clinical examination.

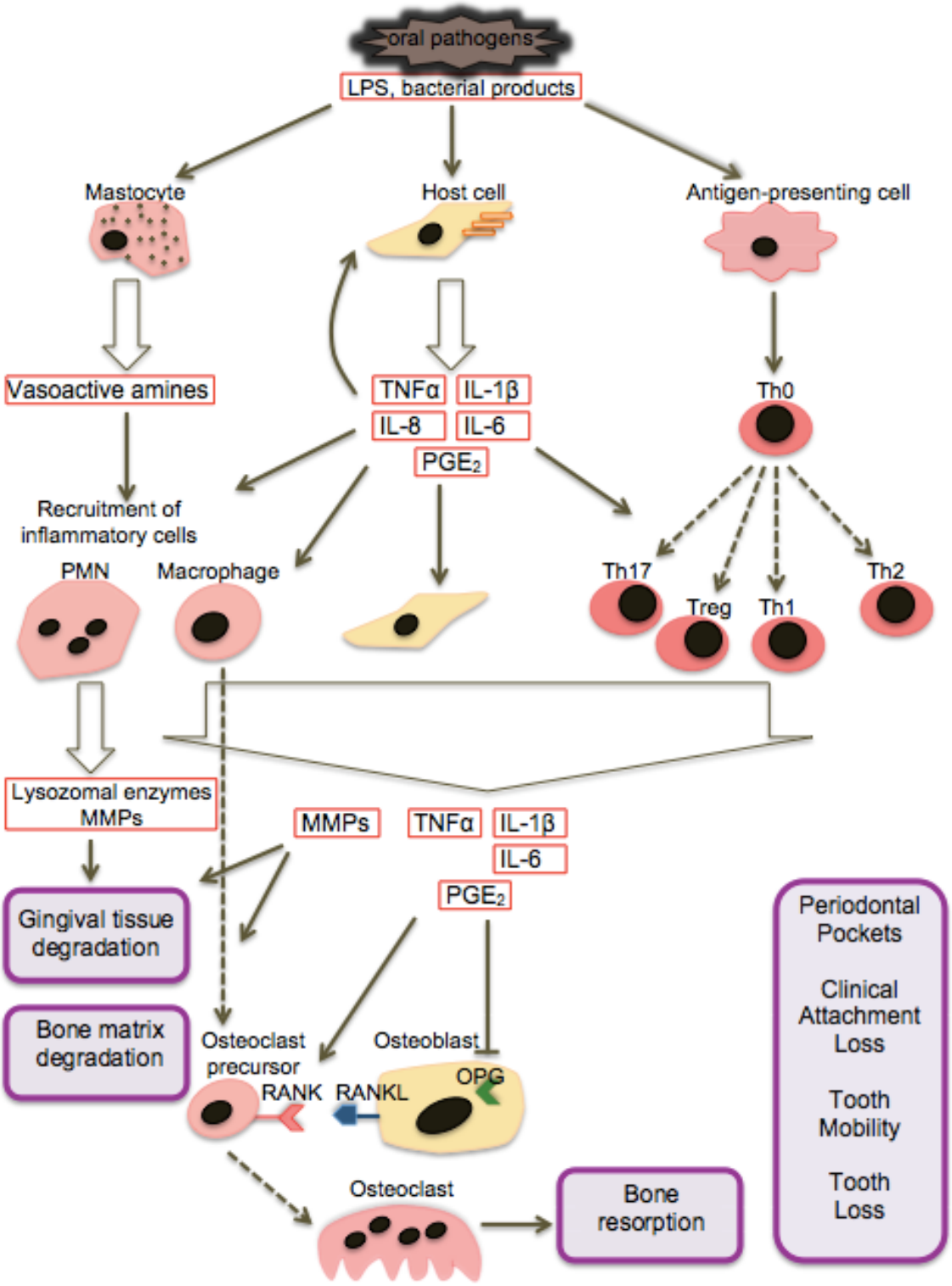

Fig. 2 Periodontal inflammation. Host-derived enzymes (MMPs) and change of osteoclast activity induced by cytokines and prostanoids are responsible for most of the soft and hard tissue destruction. Figure modified after (Yucel-Lindberg and Bage, 2013).

1.4 Matrix metalloproteinases

1.4.1 Classification and structure of MMPs

Matrix metalloproteinases form a family of secreted or cell surface-connected calcium-dependent, zinc-containing endopeptidases, which have similar structural form and can degrade extracellular matrix (ECM), basement membrane as well as numerous non-matrix substrates (Verma and Hansch, 2007, Goncalves *et al*., 2013). The first metalloproteinase was discovered in 1962, in the tail of the metamorphosing tadpole (Gross and Lapiere, 1962). Human MMPs are proteolytic enzymes responsible for the degradation of most ECM proteins during physiological organogenesis, growth, apoptosis, bone remodelling and wound repair as well as pathological processes like inflammatory diseases, including periodontal disease, caries, rheumatoid arthritis, neoplastic growth and metastases (Amalinei *et al*., 2010, Chaussain-Miller *et al*., 2006). Additionally they influence cellular proliferation, chemotaxis and cell migration (McCawley and Matrisian, 2001, Visse and Nagase, 2003). MMPs can be classified into six groups of enzymes (see Table II) (Verma and Hansch, 2007).

Table II: Classification of matrix metalloproteinase enzymes

Class	**Enzymes**
Collagenases	MMP-1, MMP-8, MMP-13
Gelatinases	MMP-2, MMP-9
Stromelysins	MMP-3, MMP-10, MMP-11
Matrilysins	MMP-7, MMP-26
Membrane-type MMPs	MMP-14, MMP-15, MMP-16, MMP-17
Other types of MMPs	MMP-12

Most of the MMPs consist of an N-terminal signal peptide or pre-domain, which is removed just after its synthesis inside the cell before secretion, and a sequence of four specific distinct domains: autoinhibitory pro-domain, catalytic domain which determines substrate specificity of MMP, hinge region (linker), and C-terminal hemopexin-like domain. The membrane-type MMPs (MT-MMPs) have an additional transmembrane domain responsible for anchoring the enzyme to the cell surface (Nagase and Woessner, 1999) (see Figure 3).

1.4.2 Regulation of matrix metalloproteinases function

MMP-8 can be synthesized as a latent zymogen by different cells; the highly glycosylated form is produced by polymorphonuclear leukocytes (PMNs), but gingival and synovial fibroblasts, epithelial cells/keratinocytes, chondrocytes, odontoblasts, oral cancer cells, monocyte/ macrophages and plasma cells are also a source of the enzyme (Sorsa *et al*., 2006).

The activity of MMP-8 in inflamed periodontium can be regulated on different levels: the positive or negative control of gene transcription, the secretion of the zymogen, its activation and inhibition and clearance (Sorsa *et al*., 2006). Growth factors, cytokines and chemical agents, can up- or downregulate the local gene expression of interstitial collagenases and their inhibitors (Nagase and Woessner, 1999). PMN-derived MMP-8 is however synthesized already during PMNs maturation in bone marrow and is stored in specific intracellular granules. Regulation through the secretion of latent pro-enzyme by degranulation takes place in response to triggering stimuli (Birkedal-Hansen, 1993). Extracellular latency of pro-enzyme is maintained by the interaction between cysteine residue in the N-terminal pro-domain and the Zn^{2+} ion at the catalytic domain. The activation follows through the disruption of this connection, called “cysteine-switch”, which converts the pro-enzyme into an active protease. It can happen either by proteolytic cleavage of the pro-domain by host or bacterial proteases or by the modification of cysteine thiol group in the pro-domain, e.g. by the reactive oxygen species which can be released from activated neutrophils (Maeda *et al*., 1998, Visse and Nagase, 2003) (see Figure 3).

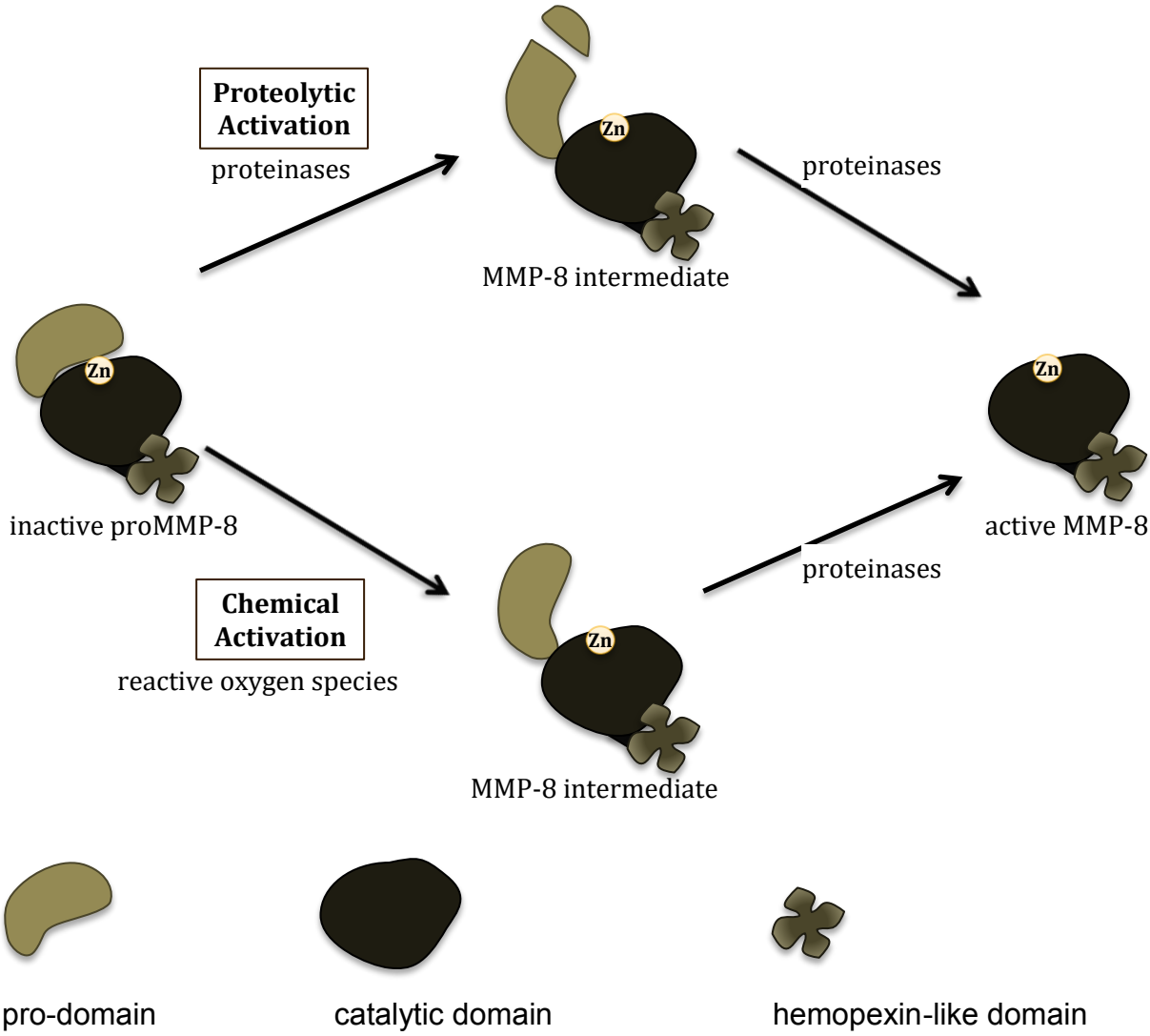

Fig. 3 The stepwise activation of proMMP-8. Proteolytic activation though cleavage of pro-domain's bait region. Chemical activation by reactive oxygen species through modification of cysteine residue of pro-domain. Both pathways lead to the removal of pro-domain and full activation of the enzyme. Drawing modified after (Van Lint and Libert, 2006).

The hemopexin-like domain is necessary for binding native collagen, but also for anchoring the tissue inhibitors of metalloproteinases (TIMPs) (Murphy and Knauper, 1997). Out of 4 members of TIMP family, the TIMP-1, 2, and 3 can reversibly block the function of active MMP-8 by slotting into the active-site cleft, as in the case of the substrate (Visse and Nagase, 2003, Thomas *et al*., 1999). The lost equilibrium between MMPs and TIMPs plays an important role in the progression of periodontitis (Sapna *et al*., 2013). While TIMPs act locally and are the key MMPs inhibitors in tissues, the main endogenous inhibitor of MMPs in tissue fluids is α2-macroglobulin. As the α2-macroglobulin/MMP complex can be removed by the endocytosis, this protein plays an important role in MMP clearance (Sternlicht and Werb, 2001). Pharmaceutical

companies make every effort to develop synthetic MMP-inhibitors which could be implemented especially in malignant and inflammatory diseases. In periodontology low-dose/sub-antimicrobial dose tetracycline-based MMP-inhibitors (LDD or SDD) showed promising results when used as adjunctives to mechanical therapy (Caton and Ryan, 2011).

1.4.3 Role of MMP-8 in periodontal disease and other inflammatory disorders

Type I collagen is the basic component of extracellular matrix in periodontal soft and hard tissues; hence its degradation is considered a crucial step in the pathophysiology of periodontal diseases (Konopka *et al*., 2012). Matrix metalloproteinases are the main proteolytic enzymes acting in periodontal tissues; MMP-8 and MMP-13 are the key collagenases, MMP-9 and MMP-14 contribute significantly to tissue degradation and other MMPs play a minor role in periodontal tissue destruction (Sapna *et al*., 2013). MMP-8 possesses the unique capacity to disrupt collagen type I and III which is essential in periodontitis but not in normal gingival tissue remodelling and is considered to be one of the key mediators of tissue destruction during inflammation of periodontal tissues (Rai *et al*., 2008, Sorsa *et al*., 2006). It is the most frequently found MMP in inflamed periodontal tissue, gingival crevicular fluid and saliva (Dejonckheere *et al*., 2011).

Oral pathogens from dental plaque can stimulate host cells to increased secretion of inflammatory mediators. Recruited neutrophils are the primary cellular source of MMP-8, and abundant inflow of neutrophils is observed in periodontal inflammation (Ozcaka *et al*., 2011). LPS stimulate macrophages which will express TNFα and MMPs (Verstappen and Von den Hoff, 2006). Their increased level in inflamed gingival tissue upregulates MMP expression in the host cells, leading to pathologically high levels of MMP-8 concentration and activity. Then a vicious circle begins, as increased levels of various proinflammatory mediators activate other cells in the periodontium, such as gingival fibroblasts, monocyte/macrophages, gingival sulcular epithelial cells/oral keratinocytes, osteoblasts/osteoclasts and endothelial cells to secrete further cytokines, proteinases and MMPs (Sorsa *et al*., 2006). The role of MMPs in the pathogenesis of periodontitis is schematically visualised in Figure 2.

MMP-8 at physiological level seems, however, to have a positive, anti-inflammatory effect, probably by processing some anti-inflammatory cytokines and chemokines (Kuula *et al*., 2009). Summing up, it is the distorted balance between MMPs and their

inhibitors (TIMPs) that can lead to the degradation of extracellular matrix of the connective tissue, basement membrane and alveolar bone and thus to signs of periodontal disease (Gursoy *et al*., 2010).

Detailed functions of particular MMPs in different diseases are still not fully understood, but they are often up-regulated in inflammatory and malignant diseases, such as asthma, atherosclerosis, myocardial rupture, head and neck squamous cell carcinoma or breast cancer (Sorsa *et al*., 2004, Dejonckheere *et al*., 2011). Multiple clinical studies were conducted investigating the involvement of MMP-8 in the development of inflammatory and neoplastic diseases, with MMP-8 being a putative drug target in those conditions (Dejonckheere *et al*., 2011). Interestingly, excessive level of MMP-8 plays a role in the progression of inflammatory response, but minimal physiological level of MMP seems to have a protective, anti-inflammatory and anti-carcinogenic function and might be of crucial significance during the recovery process (Sorsa *et al*., 2006).

1.4.4 Role of MMP-blockers in the treatment of periodontitis and other inflammatory diseases

Due to the significant role of MMPs in the pathogenesis of inflammatory diseases and cancer progression numerous studies concentrating on the enzyme as a potential drug target have been conducted. However, TIMPs as natural inhibitors proved to be rather insufficient in reducing the MMP activity and unselective in their inhibition (Overall and Lopez-Otin, 2002). The first MMP inhibitors accepted for the clinical trial in the treatment of neoplasms were marimastat and batimastat, their MMP inhibitory effect was based on chelation but the results were disappointing (Coussens *et al*., 2002). In arthritis and periodontitis, the exaggerated MMP inhibition led to an aggravation rather than to an improvement of the clinical status (Coussens *et al*., 2002, Bjornsson *et al*., 2004).

Not fully blocking, “leaky” MMP-inhibitors, based on tetracyclines, are safer and more effective, as they reduce pathologically elevated levels of MMPs, but do not go beyond the physiologically essential concentration (Sorsa *et al*., 2006). Currently, the only collagenase inhibitor accepted by the Food and Drug Administration (FDA) for the treatment of periodontal diseases is doxycycline hyclate, Periostat® (PMRS, Inc. Horsham, PA 19044 USA). It is a low-dose/ sub-antimicrobial dose of doxycycline medication (LDD or SDD) and can be used intraorally as an adjunctive to subgingival debridement in periodontitis patients. Its therapeutic effect is basically due to the modulation of host response. Periostat® does not have an antibacterial effect and does

not lead to the formation of bacterial resistance, and can be administered for up to 9 months (Food and Drug Administration, 2003). SDD significantly improved the clinical results contributing to the gain of clinical attachment and the reduction of periodontal pocket depths, compared with the debridement alone (Preshaw *et al*., 2004a). Preliminary data show the potential usefulness of MMP inhibitors in the treatment of patients with peri-implantitis and with referrals for adjunctive periodontal surgery (Honibald *et al*., 2012).

1.5 Diagnostic tools for periodontal disease

1.5.1 Classical methods

Current diagnostic methods are still based mainly on clinical parameters introduced more than 50 years ago: pocket probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP) and tooth mobility measured with calibrated periodontal probe and alveolar bone level assessed from radiological findings (Armitage, 2004b, Giannobile *et al*., 2009). Additionally, Papilla Bleeding Index (PBI) and Approximal space Plaque Index (API) provide information about patient's oral hygiene habits and the main local etiological factor of periodontitis, the microbial dental plaque (Saxer and Muhlemann, 1975, Lange, 1977). It is assumed that pathologically deepened periodontal pockets as well as bleeding on probing are signs of unstable periodontium. Therefore a subgingival debridement should be performed repeatedly to prevent tissue loss and disease progression (Renvert and Persson, 2004). These classical methods of diagnosing periodontitis are limited to determining the history of periodontal destruction, which is the result of disease course until the time of measurement, rather than predicting the future disease activity (Kraft-Neumarker *et al*., 2012). BOP may indicate the periodontal disease activity but absence of bleeding is a more specific negative predictor of periodontitis progression (Lang *et al*., 1986, Lang *et al*., 1990). Hence, standard clinical parameters have very limited prognostic value leading possibly to the undertreatment of some patients (tooth loss or general health complications when the adequate treatment was delayed) or to the overtreatment of other patients (recessions, tooth hypersensitivity, root caries risk, as a result of frequently repeated mechanical treatment) (Reiker *et al*., 1999, Sykes, 2007, Renvert and Persson, 2002).

1.5.2 Biomarkers of periodontal disease

Clinical examination and periodontal measurements describe previous irreversible periodontal tissue destruction. They can provide information about the activity of the disease only if the same measurements are taken repeatedly at different time-points and the difference between two measurements is assessed (Armitage, 2004a). Hence, periodontal research puts a lot of efforts into discovering methods which could predict the future course of periodontal disease or at least determine the activity level of the current disease at a given time-point. Such ideal diagnostic method or marker should have high sensitivity and specificity for screening periodontally susceptible subjects in large populations, it should be able to distinguish patients with disease progression from those with a diseased but stable status, active sites from inactive ones, to predict tissue destruction in particular patients and sites and to monitor response to the applied therapy (Buduneli and Kinane, 2011).

A biomarker (biological marker) is a substance “that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group, 2001). Putative periodontal bacterial levels in subgingival plaque may point out the sites or patients with increased risk of periodontitis progression but it is the molecules closely linked to bone and soft tissue destruction that are the promising candidates for biomarkers of periodontal diseases (Byrne *et al*., 2009, Buduneli and Kinane, 2011, Kinney *et al*., 2014). Biomarkers can be derived directly from inflamed periodontal tissue during biopsy, from oral fluids, such as gingival crevicular fluid (GCF), peri-implant sulcular fluid (PISF), mouth-rinse and saliva or blood circulatory system - serum or plasma. GCF and saliva are particularly suitable, as they can be easily and noninvasively collected and comprise both locally and systematically synthesized molecules (Buduneli and Kinane, 2011).

1.5.2.1 Gingival crevicular fluid as a source of diagnostic markers for the prediction of periodontal breakdown

GCF is a transudate from blood vessels in the gingival connective tissue that enters the sulcus through crevicular epithelium and contains additionally resident host cells and microorganisms from the microbial dental plaque and their inflammatory mediators and tissue breakdown products (Buduneli and Kinane, 2011, American Academy of Periodontology, 2012b). As its composition depends on the interplay between bacterial

biofilm and host cell response and mirrors current processes in periodontium, it can serve as a source of potential prognostic markers for the progression of periodontitis (Reinhardt *et al*., 2010, Kinney *et al*., 2014). Over 65 GCF components were assessed as potential markers for the progression of periodontitis (for overview see Table III) (Gupta, 2012, Gupta, 2013). They can be divided into three groups:

- host-derived enzymes and their inhibitors
- tissue breakdown products
- inflammatory mediators and host-response modifiers.

At present, there are no real indicators which could help clinicians to determine the sites of high risk for progression before the actual damage occurs, therefore biomarker testing could be beneficial, especially for patients in the maintenance phase of therapy (Armitage, 2004a).

Table III: Periodontitis progression biomarkers in GCF, after (Gupta, 2012, Gupta, 2013, Armitage, 2004a)

Host-derived enzymes and their inhibitors

- Aspartate aminotransferase
- Alkaline phosphatase
- Acid phosphatase
- β-Glucuronidase
- Elastase
- Elastase inhibitors
  - α_2 – Macroglobulin
  - α_1 - Proteinase inhibitor
- Cathepsins
  - Cysteine proteinases (B, H, L)
  - Serine proteinase (G)
  - Cathepsin D
- Trypsin-like enzymes
- Immunoglobulin-degrading enzymes
- Dipeptidyl peptidases
- Nonspecific neutral proteinases
- Collagenases
  - Matrix metalloproteinase-1 (MMP-1)
  - Matrix metalloproteinase-3 (MMP-3)
  - Matrix metalloproteinase-8 (MMP-8)
  - Matrix metalloproteinase-13 (MMP-13)
- Gelatinases
  - Matrix metalloproteinase-2 (MMP-2)
  - Matrix metalloproteinase-9 (MMP-9)
- Tissue inhibitor of MMP-1 (TIMP-1)
- Stromyelysins
- Myeloperoxidases
- Lactate dehydrogenase
- Arylsulfatase
- β-N-acetyl-hexosaminidase

Tissue breakdown products

- Glycosaminoglycans
  - Hyaluronic acid
  - Chondroitin-4-sulfate
  - Chondroitin-6-sulfate
  - Dermatan sulfate
- Hydroxyproline
- Fibronectin fragments
- Connective tissue and bone proteins
  - Osteonectin
  - Osteocalcin
  - Type I collagen peptides
  - Osteopontin
- Laminin
- Calprotectin
- Hemoglobin β-chain peptides
- Pyridinoline crosslinks (ICTP)
- Polypeptide growth factors

Inflammatory mediators and host-response modifiers

- Cytokines
  - Interleukin -1α
  - Interleukin -1β
  - Interleukin -1ra
  - Interleukin-2
  - Interleukin-6
  - Interleukin-8
  - Tumor necrosis factor α
  - Interferon α
- Prostaglandin E_2
- Leukotriene B_4
- Acute-phase proteins
  - Lactoferrin
  - Transferrin
  - α_2-Macroglobulin
  - α_1-Proteinase inhibitor
  - C-reactive protein
- Autoantibodies
  - Anti-desmosomal antibody
- Antibacterial antibodies
  - IgG_1, IgG_2 , IgG_3 , IgG_4, IgM, IgA
- Plasminogen activator (PA)
- PA inhibitor-2 (PAI-2)
- Substance P
- Vasoactive intestinal peptide
- Neurokinin A
- Neopterin
- Platelet -Activating Factor
- CD14
- Cystatins
- Calgranulin A (MRP-8)

1.5.2.2 MMP-8 as a biomarker for periodontitis

Active MMP-8 originating from the neutrophils is the main host cell-derived collagenase causing periodontal tissue degradation (Lee *et al*., 1995). At the site level, the MMP-8 values sampled in GCF can differentiate the healthy sites from those affected by gingivitis and periodontitis (Mäntylä et al., 2003, Prescher et al., 2007). At the patient level, high levels of MMP-8 sampled from saliva also correlated with clinical signs of periodontitis and radiological bone loss in those patients (Salminen *et al*., 2014, Gursoy *et al*., 2013, Rai *et al*., 2008). Increased level of MMP-8 was also observed in the plasma of patients with chronic periodontitis (Marcaccini *et al*., 2009).

The level of activation of MMP-8 in GCF correlates positively with the severity of periodontal disease (Romanelli *et al*., 1999, Leppilahti *et al*., 2014a). Oral rinse samples from patients with strongest inflammatory burden, e.g. with multiple and deep periodontal pockets and more BOP, showed higher levels of MMP-8 than those from patients with lower inflammatory status (Leppilahti *et al*., 2011).

Successful periodontal treatment in form of scaling and root planing reduces probing depth, clinical attachment loss and bleeding on probing as well as mean MMP-8 concentration in GCF (Mäntylä et al., 2006, Kinane et al., 2003, Marcaccini et al., 2010). Even more improvement in clinical parameters and stronger reduction of GCF MMP-8 levels could be observed in patients who were administered azithromycin or subantimicrobial doses of doxycycline additionally to SRP (Tuter *et al*., 2010, Emingil *et al*., 2012).

MMP-8 levels can identify sites or patients who are at risk of periodontitis progression or have poor response to standard treatment (Leppilahti *et al*., 2015). Particularly elevated concentrations of MMP-8 were observed in sites which did not improve after SRP in smokers, the MMP-8 concentration in those sites remained persistently high at the subsequent visits (Mäntylä et al., 2006). In continuously active sites, the level of MMP-8 did not show significant decrease after treatment, in contrast to inactive sites (Hernandez *et al*., 2010). Activity or baseline concentration of MMP-8 was also higher in patients with progressive destruction of periodontium, and there was an increase of activity of MMP-8 with time in those subjects, compared to patients with non-progressive status (Lee *et al*., 1995, Kinney *et al*., 2014).

1.5.2.3 Development of laboratory and chair-side diagnostics for periodontitis based on MMP-8 testing

In laboratory conditions patient's body tissue or body fluid samples can be analysed for the periodontitis biomarkers without any difficulties. Multiple studies performed on the increased level of MMP-8 in affected tissues and body fluids of patients with periodontitis indicate a potential role of MMP-8 as a biomarker for diagnosis and monitoring of periodontitis (Sapna *et al*., 2013). Saliva is a convenient source for patients' screening and disease course monitoring at the patient level whereas GCF represents a suitable material for the detection of particular tooth or implant sites which might be on the verge of tissue breakdown. There is a need for a handy, chair-side, point-of-care test used for diagnosing and monitoring periodontal disease in medical practice settings (Sorsa *et al*., 2004, Uitto *et al*., 2003). MMP-8, appraised as a potential candidate for such a test was launched on the market both for laboratory and rapid chair-side testing (Kiili *et al*., 2002, Sorsa *et al*., 2004, Izadi Borujeni *et al*., 2015).

Global information on the periodontal tissue breakdown marked by the aMMP-8 level can be obtained from the oral-rinse and is commercially available as PerioSafe® (Dentognostics GmbH, Jena, Germany) or Perio-Marker® aMMP-Schnelltest, distributed until 2012 by Chlorhexamed® (GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Bühl, Germany), later taken over by Miradent (Hager & Werken GmbH & Co. KG, Duisburg, Germany) (Izadi Borujeni *et al*., 2015, Heikkinen *et al*., 2015). It is approved in Germany since 2010, resembles a pregnancy test based on lateral-flow-sandwich-immunoassays with specific monoclonal antibodies, and gives a simplified qualitative yes/no result whether or not the clinically relevant level of 25 ng/mL is exceeded (Firla, 2012). Later, a mouth-rinse-based rapid test (PerioSafe® Home, Dentognostics GmbH, Jena, Germany) for over-the-counter distribution was launched for self-testing of collagenolytic activity in the oral cavity at home.

A site-specific analysis of collagenolytic activity measured by the level of MMP-8 can be performed with various methods: in the laboratory setting by a time-resolved immunofluorometric assay (IFMA) or Enzyme Linked Immunosorbent Assay (ELISA) which use specific monoclonal antibodies, 8708 and 8706 (Medix Biochemica Oy, Kauniainen, Finland), to detect the active form of MMP-8, or the commercially available ELISA kits, which cannot distinguish between different MMP-8 forms such as Human Total MMP-8 Quantikine ELISA Kit (Quantikine R&D Systems Inc., Minneapolis, MN, USA) (Kraft-Neumarker *et al*., 2012, Sorsa *et al*., 2010, Konopka *et al*., 2012). In the

dental or general medical office setting, a dentoAnalyzer device based on the sandwich-based immunoassay system or an MMP-8 specific immunochromatographic chair-side dip-stick test was implemented (Sorsa *et al*., 2010). At the time of this manuscript preparation, some of the above mentioned methods were not available anymore and were substituted by further products or their updated versions: ELISA aMMP-8 laboratory tests for precise quantitative results of aMMP-8 levels in GCF or PISF are performed after samples are submitted to the central laboratory in Jena (Dentognostics GmbH, Jena, Germany) and qualitative chair-side tests ImplantMarker® or ImplantSafe, based on lateral-flow sandwich immunoassays with specific monoclonal antibodies (Hager & Werken GmbH & Co. KG, Duisburg, Germany and Dentognostics GmbH, Jena, Germany, respectively), are indicated for single-site sampling around implants (Dentognostics, 2015).

The demand for a better diagnostic tool for the detection of periodontitis has not been fulfilled yet, therefore there is a dynamic development of further complementary chair-side tests in the biochemical market sector. However, the real challenge seems to be not the change from the laboratory bench to chair-side diagnostics, but rather the incorporation of biomarker testing into everyday clinical practice (Giannobile *et al*., 2011).

2. Objectives of the study

The primary objective of the study was to determine if levels of GCF aMMP-8 sampled from a limited number of sites can predict disease progression (relapse) during supportive periodontal therapy of periodontally compromised patients, adopting various definitions of patient-based progression of disease.

Secondary goals were to correlate GCF aMMP-8 levels with clinical periodontal parameters: PD and BOP, measured at the same time point (expressing the severity of disease) at the site level. Further, patient-based changes of clinical parameters: Plaque Index (PI), PD, CAL and BOP during consecutive visits following initial periodontal therapy with or without adjunctive systemic antibiotics, were analysed, as well as site-based GCF aMMP-8 levels at consecutive visits.

My working hypotheses are: I want to prove that GCF aMMP-8 levels pooled from four sites per patient analysed with ELISA do predict disease progression (relapse) at the follow-up visit, using for that purpose various definitions of patient-based disease

progression. Next, I want to prove that GCF aMMP-8 levels correlate with PD and BOP from the same sites, measured with an electronic constant-force periodontal probe. Furthermore, I want to prove that PI, PD, CAL, and BOP analysed at the patient-level as well as GCF aMMP-8 levels decrease during consecutive visits following initial non-surgical periodontal therapy.

3. Materials & Methods

3.1 Study subjects

Patients' contact data were obtained from the Charité internal, administrative patients' database "Parobase" of "CharitéCentrum 3 für Zahn-, Mund- und Kieferheilkunde". About 200 patients, who were previously diagnosed witch chronic or aggressive form of periodontitis, were contacted preferably by phone or, when the phone number was not available or, when patients repeatedly did not respond to an invitation, by post card. Additionally, patients referred from colleagues in the Department of Restorative Dentistry and Periodontology or from the emergency room and from new patients' pool were offered a consultation appointment, where the inclusion and exclusion criteria were verified. In the inclusion criteria, there was no differentiation between chronic and aggressive periodontitis patients, provided that the patients presented with moderate to severe form of the disease. Between December 2008 and September 2009, a total of 71 patients were recruited for the multi-centre, double-blind, randomised, placebo controlled, phase IV trial "Adjunctive Antibiotic Therapy of Periodontitis: Long-Term Efficacy on Disease Progression and Oral Microbial Colonization" (ABPARO-study, study No: EH 365/1-1, EudraCT-Nr: 2006-005854-61), coordinated by Prof. Ehmke (Policlinic for Periodontology, University Clinic, Münster) (Harks *et al*., 2015). Subjects formed the patient group for the study centre in Berlin, if they fulfilled the criteria presented in Table IV; all the patients were automatically recruited for the sub-study „Investigation of the change of the rheumatoid arthritis biomarkers' level during periodontal therapy" conducted at the Department of Restorative Dentistry and Periodontology, Charité University Clinic in Berlin.

From the above group of patients, 34 subjects underwent a measurement of the enzyme active Matrix Metalloproteinase 8, generating the databank **"**Determination of active Matrix-Metalloproteinase 8 (aMMP-8) levels in the Gingival Crevicular Fluid as a Diagnostic Test during Periodontal Maintenance Therapy". Included were all patients involved in the main study who, during the time of a first MPP-8 sampling (October

2009) had not reached the stage of an initial periodontal therapy (supra- and subgingival debridement, removal of supra- und subgingival calculus and biofilm). Three patients were excluded from statistical analysis as they discontinued their participation in the study before the second sampling; hence the data were not suitable for the analysis (see Figure 4).

Table IV: Subject inclusion and exclusion criteria from the main study:

Subject Inclusion Criteria:	Subject Exclusion Criteria:
PSI Grade IV in at least one sextant 18 - 75 years old clinical and radiological signs of moderate to severe chronic or aggressive periodontitis at least 10 natural teeth in situ PD ≥ 6mm at min. 4 teeth willingness to participate and time availability abstaining from using antimicrobial mouth-rinses except when prescribed by study investigator informed consent signed sufficient knowledge of German	confirmed or assumed allergies or hypersensitive skin reactions to amoxicillin, metronidazole or confirmed lactose intolerance Down syndrome AIDS/HIV regular intake of systemic medication affecting periodontal conditions e.g. phenytoin, nifedipin, cyclosporine A or steroid drugs antibiotic treatment during dental appointments required extensive dental treatment required professional subgingival periodontal therapy during 6 months prior to baseline pregnancy/ breastfeeding rampant caries intra- or perioral piercings dental students or dental professionals participation in clinical dental trial within 6 months preceding the study cognitive deficits

PSI = Periodontal Screening Index; PD = pocket depth

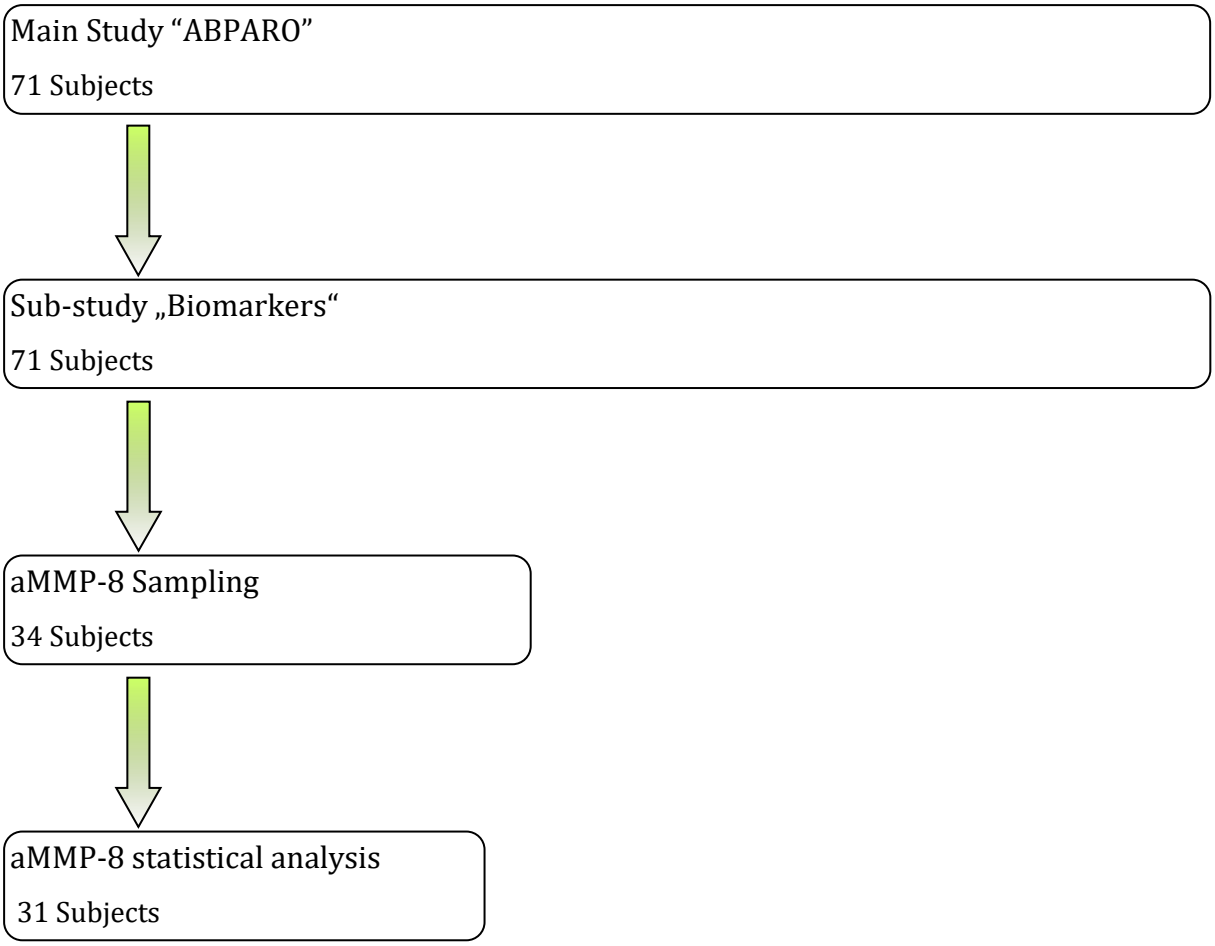

Fig. 4 Diagram showing patients' recruitment for the analysis of the enzyme aMMP-8.

Patients were informed orally and in writing about their disease, treatment options and study protocol following the "Good Clinical Practice" standard (ICH-GCP, European Medicines Agency, 2002). Patients were incorporated into the main study regime either directly after screening and after signed the informed consent form approved by the Ethics Committee of the Medical Council, or the standard pre-treatment was conducted first and the patients decided afterwards if they wished to be treated within the study project.

A subject discontinued the study if:

- s/he violated the protocol or did not want to follow it
- did not keep the appointment
- was found to have serious adverse reactions to the medications prescribed

3.2 Trial design and aim of the study

The investigation was designed as a substudy to the double-blind, parallel group, randomised, placebo-controlled trial over a total study period of 38-months. 71 patients were registered for the study not later than at visit 1. Twelve visits were required for each patient, with additional pre-treatment visits, if such measures were needed, according to standard recommendations before initiating periodontal treatment. The participating patients were stratified into four groups depending on the extent of periodontal disease and smoking habit at visit 2, as well as randomised for a test (adjunctive antibiotic therapy) or control (placebo drug) group. Baseline clinical measurements were taken, future aMMP-8 sites were assigned and first sampling took place. Within the following 6 weeks, at visit 3, both test and control group patients received the same standard periodontal therapy (mechanical supra- and subgingival debridement in two or, if feasible, one session) and 14 days later baseline aMMP-8 samples were collected. Re-evaluation was undertaken approximately 8 weeks later at visit 4, which involved clinical examination and aMMP-8 sampling. Supportive periodontal therapy, including clinical examination, mechanical debridement and aMMP-8 sampling, began approximately 12 weeks later at visit 5. Clinical examination and treatment were offered at the following visit no. 6, when last data for the statistical analysis were collected. Patients stayed in the further supportive periodontal therapy until visit 12, when examinations and treatment were conducted as part of main study design. Flow diagram of trial design, with main procedures and stages including aMMP-8 sampling, is shown in Figure 5. Exact course of action is described in the following paragraphs.

3.3 Subject registration, randomisation and stratification

Subjects fulfilling all inclusion criteria, not meeting exclusion criteria and having signed the informed consent form were registered into the study after filling the Case Report Form during visit 1. The main study coordinating centre in Münster was informed about the registration per fax. The subjects were assigned a three-digit registration number (counting backwards from 999). At visit 2 the patients were randomised according to the disease severity and the smoking habit and assigned to one of the four strata.

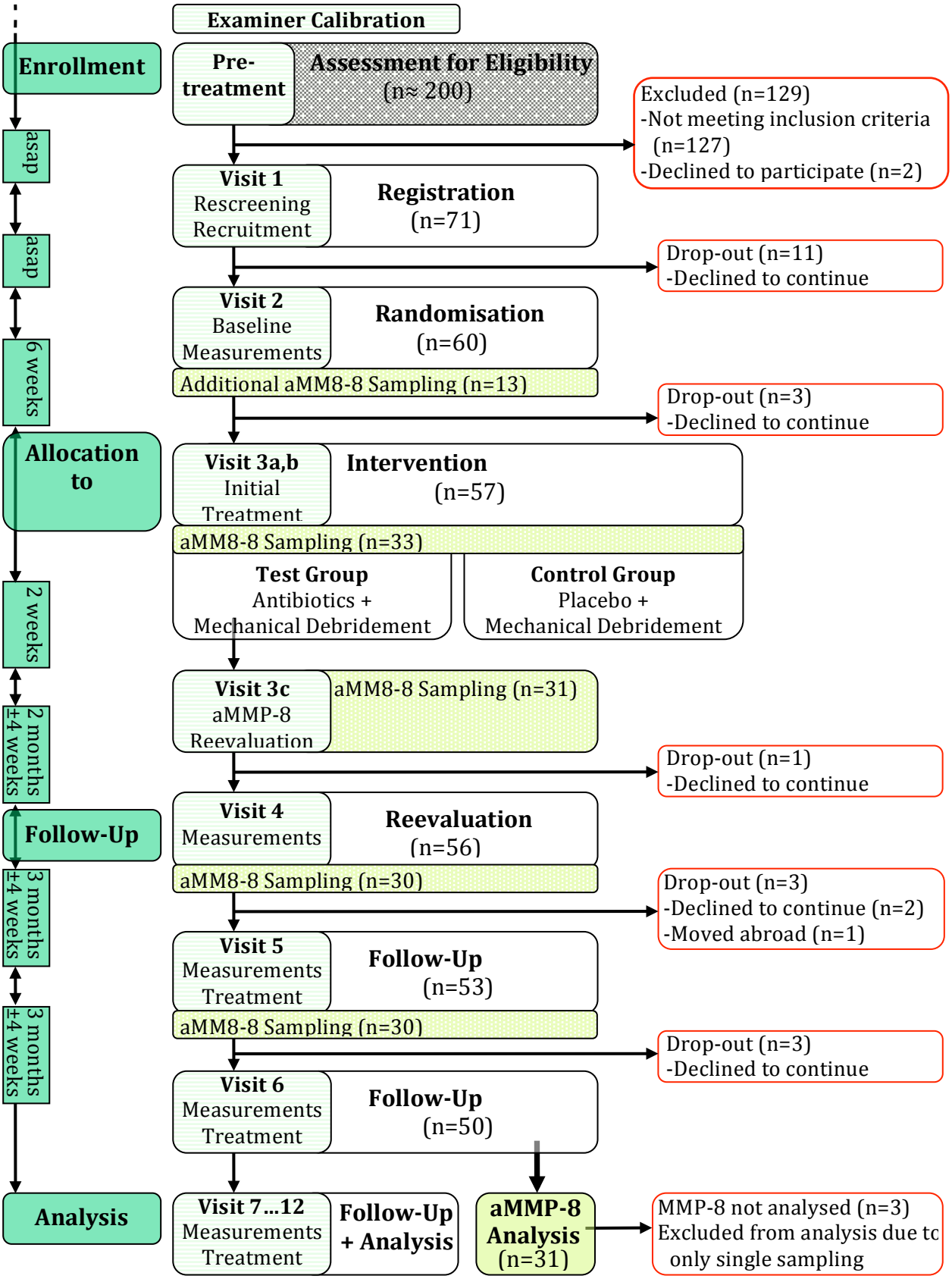

Fig. 5 Trial design of main study until visit 6, supplemented with aMMP-8 measurements.

3.4 Clinical procedures

3.4.1 Study activity chart

Detailed study activity chart of the main study as well as additional data collected for the sub-study including aMMP-8 sampling are shown in Table V

Table V: Study activity chart

Visit:	**1**	**1a**	**2**	**3a**	**3b**	**3c**	**4**	**5**	**6**	**7-**
Recruitment										
Periodontal Screening	X									
Medical Health History	X	X	X	X	X	X	X	X	X	...
Inclusion/Exclusion criteria	X									
Study Information	X									
Informed consent/ Registration	X									
Randomisation/Balancing			X							
Treatment Activities										
Oral Hygiene Instructions		X	X				X	X	X	...
Supragingival Debridement				X	X			X	X	...
Subgingival Debridement				X	X			X	X	...
Drug Dispense/ Return					X	X				
Examinations										
Plaque Index			X				X	X	X	...
Relative Attachment Level			X				X	X	X	...
Bleeding On Probing			X				X	X	X	...
Pocket Probing Depth			X				X	X	X	...
Gingival Recessions			X				X	X	X	...
Furcation Involvement			X				X	X	X	
Clinical Inspection			X	X	X	X	X	X	X	...
Occlusal inspection							X	X	X	...
Mobility			X							...
AE/SAE			X	X	X	X	X	X	X	...
Periodontal Abscess			X	X	X	X	X	X	X	...
X-Rays			X							
Intraoral Photographs			X							...
Microbial Samples										
Four Sample Teeth			X				X	X		...
GCF Samples (Periotron)		X					X	X		
Blood Samples										
Rheuma Saliva+Blood Quick-Test	X									
Rheuma PAX	X									
Full blood, Plasma, Serum	X		X				X		X	...
DNA Storage Card										...
Smoking- CO Measurements			X				X			
Questionnaires										
PSQ, OHIP, FAZ-LQ			X				X		X	...
HADS, SF-36			X							...
ZUF-8							X			...
BMI Measurements	X									
Nutrition Questionnaire	X									
aMMP-8 Sampling			X	X		X	X	X		

3.4.2 Oral hygiene phase

Depending on patient's individual needs, 2 to 4 pre-treatment visits took place, where oral hygiene instruction, oral hygiene motivation and professional tooth cleaning were performed. Supra- and epigingival calculus, biofilm and discolorations were removed with universal scalers and curettes (M23, M23A, GX4, Deppeler SA, Switzerland) and an airscaler (SONICflex 2003 L, KaVo Dental GmbH, Germany) or ultrasonic handpiece (SIROSONIC L, Sirona Dental Systems GmbH, Germany). If hard extrinsic discolorations were present, an air polisher was additionally used (PROPHYflex 2, KaVo Dental GmbH, Germany) with an air polishing powder (Air-Flow Powder Classic, EMS, Switzerland). All the tooth surfaces were polished with a rubber cup (Prophy Cup, Kerr, USA) and polishing pastes (Proxyt, Ivoclar Vivadent GmbH, Germany). An appropriate tooth cleaning technique was demonstrated, the patient was trained how to use it, with a special attention to the cleaning of interdental spaces. Initial oral hygiene status and hygiene-dependent gingival inflammation level was captured by the Approximal space Plaque Index (API) (Lange, 1977) and the Papilla Bleeding Index (Saxer and Muhlemann, 1975). During the initial therapy (hygiene phase) a very good level of oral hygiene (API ≤ 25 % and PBI ≤ 12 as a goal) had to be achieved before the patients were allowed to undergo the baseline measurements and an active treatment. Further pre-treatment was performed during subsequent appointments at the Charité University Clinic; optionally, the patients were referred to their home dental practitioners if they preferred. During pre-treatment, if indicated, new fillings were provided or recontoured, polished and overhanging crown margins were removed. Root canal treatments were performed where necessary. Teeth with circular attachment loss and less than 2 mm remaining periodontal support were classified as hopeless and extracted; if necessary, a long-term fixed or removable dental prosthesis was provided. As soon as the dental and periodontal pre-treatment was completed and a satisfactory hygiene level was achieved, the patients were offered an appointment for study visit 1. Alternatively, as mentioned before, pre-treatment took place directly after completing visit 1.

3.4.3 Sequence of trial periods

3.4.3.1 Visit 1: rescreening and allocation

After successful completion of the pre-treatment phase, the patients were rescreened at 6 sites per tooth using the periodontal screening index (PSI), and rescreened for

inclusion and exclusion criteria, the medical history was captured and the data registered on the Case Report Form (CRF). The smoking status was established (less than one, one, two, more than two packs of cigarettes per day, or other tobacco consumption). Venous blood samples were collected from the superficial veins of the forearm in the antecubital fossa and processed in the in-house laboratory or shipped immediately for further processing in gusseted wallets to the study clinical chemistry laboratory of the University of Greifswald, in accordance with the study protocol. Intraoral hard and soft tissue status was recorded on a digital photo camera for comparison of occlusal relief during the entire study. An individual study number was assigned.

3.4.3.2 Visit 2: baseline measurements, randomisation and aMMP-8 site assignment

1. Measurements

During visits 2, 4, 5 and 6, the following clinical parameters were measured in each patient: Plaque Index, relative attachment level, clinical attachment level, bleeding on probing, pocket probing depth, gingival recessions, furcation involvement, mobility. After assessment of the Plaque Index (O'Leary *et al*., 1972) for all teeth, further clinical measurements took place with the help of a highly inter- and intra-operator reproducible Florida disk probe® handpiece **(**Florida Probe FP 32 with Software Version 6.6.2, Florida Probe Corporation, USA), which was set on the 0.2 mm accuracy and calibrated for 0.25 N pressure:

- relative attachment level (RAL) – six sites per tooth were measured in two series, potential differences greater than 1 mm between two time points were remeasured, when possible, and corrected,
- bleeding on probing (BOP) was registered as present or absent, in each quadrant approximately 30 seconds after the first course of RAL measurements.

Probing pocket depths (PD) and gingival recessions (Rec) were registered with a standard Florida probe® handpiece. Probing pocket depths and recessions were added later on mathematically to calculate the clinical attachment level (CAL).

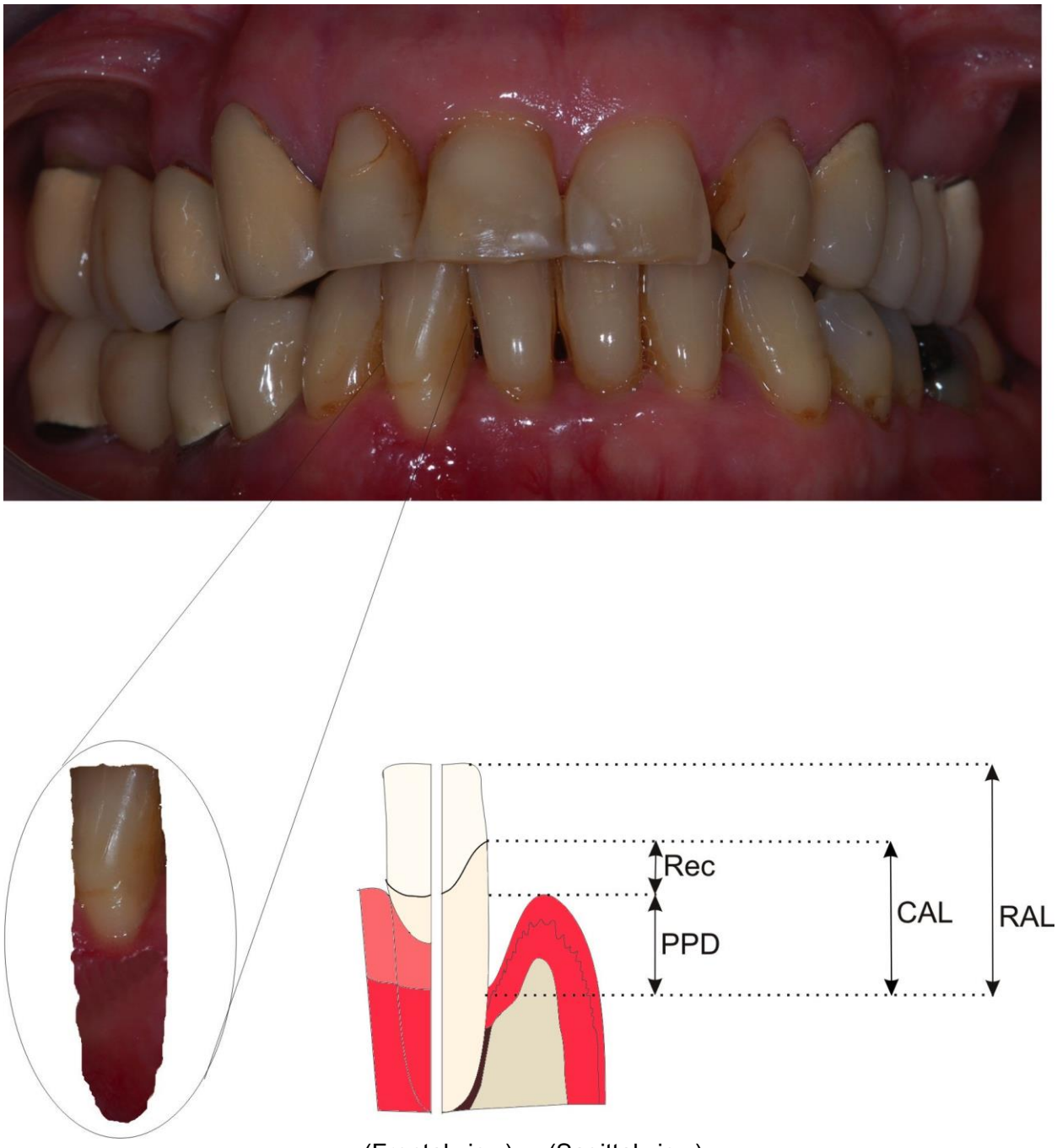

Fig. 6 Illustration of clinical parameters examined: PD = probing pocket depths; Rec = gingival recessions; CAL = clinical attachment level; RAL = relative attachment level.

Horizontal furcation involvement was estimated on a 0-3 scale with a manual furcation probe (Nabers Probe). Tooth mobility was assessed with the help of a hand instrument on a 0-III scale. All the measurements were performed by a calibrated clinician who was blinded to the study medication. Clinical measurements and their reference points are shown schematically using the example of one tooth in Figure 6.

Smoking habits were determined by a chair-side measurement of carbon monoxide concentration in exhaled air (Bedfont-Smokerlyzer, Bedfont Scientific Ltd, UK).

Intraoral radiographs, if no current ones were available, were taken in a paralleling technique and all questionnaires were completed.

2. Stratification, randomisation and microbiological sample tooth selection

Using pre-defined randomisation tables, subjects were stratified according to the extent of periodontal disease and smoking habit into one of four strata, as seen in Table VI.

Table VI: Patients' division into four strata depending on the severity of periodontal disease and smoking intensity

Stratum 1	Stratum 2
PD ≥ 6 mm at < 38% of the teeth non-/light smoker: <7 ppm	PD ≥ 6 mm at ≥ 38% of the teeth non-/light smoker: <7 ppm
Stratum 3	**Stratum 4**
PD ≥ 6 mm at < 38% of the teeth moderate/heavy smoker: ≥7 ppm	PD ≥ 6 mm or more at ≥ 38% of the teeth moderate/heavy smoker: ≥7 ppm

PD = probing pocket depths

For the needs of microbiological analysis four teeth with a PD ≥ 6 mm were selected per patient, evenly distributed in the mouth, if possible, and samples were repeatedly taken during the course of study.

3. Microbiological and blood samples

Pooled subgingival plaque samples were taken from the four sample sites for microbiological analysis with sterile paper points. Samples were sent to the coordinating centre in Münster after being stored in a refrigerator at +4°C.

Venous blood samples (1 x 10 mL full blood for the main study, 1 x 10 mL full blood, 2 x plasma and 2 x serum samples for the sub-study) were taken. One full blood sample was sent as soon as possible to the coordinating laboratory in Greifswald and the remaining samples were processed in the in-house laboratory: centrifuged, aliquoted and frozen at -80°C after labelling with patient's code number for later analysis.

4. aMMP-8 site assignment and sampling

For each study patient, four tooth sites at four different teeth were selected, each with a PD of at least 4 mm, according to the basement measurement and thus classified clinically as periodontally involved sites. It was aimed to have an even distribution throughout the mouth by choosing one site per quadrant, and in turn mesial and distal sites as well as buccal and lingual ones, if appropriate teeth were detected. Selected surfaces were recorded in study chart and an individual code number was assigned to each sample consisting of patient's code number, visit number and consecutive number from 1 to 4.

The aMMP-8 measurement in four gingival sulci was accomplished with test strips (GCF Collection Strips, Dentognostics GmbH, Germany). According to manufacturer's recommendations, after isolation with cotton rolls and slightly drying the surrounding area, the final 2 mm of the stripe were immersed into the investigated periodontal pocket between the tooth surface and gingival margin and left for 30 seconds each. In case of significant blood contamination, the sample was discarded and the procedure was repeated. Individual samples were placed into empty 1.5 mL test tubes, sealed and sent to the cooperating laboratory in Jena (Dentognostics GmbH, Germany) together with a shipping form. Further processing for the quantitative analysis of aMMP-8 samples took place in the laboratory using enzyme linked immunosorbent assay (ELISA) capable of detecting mostly active form of the enzyme thanks to specific antibodies used, and the results were delivered via electronic mail. The workflow of handling of aMMP-8 samples is depicted in Figure 7. Samples were collected during visits 3a, 3c, 4 and 5. As the collection of aMMP-8 samples was initially not feasible during visit 2 due to financial limitations, a limited amount of samples from this period could be obtained additionally due to the drop-out of other patients.

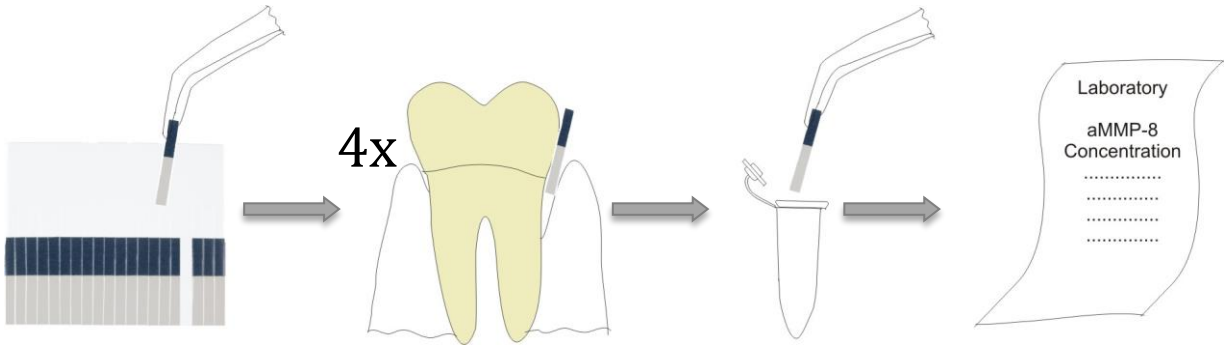

Fig. 7 Sampling of aMMP-8 with test strips from four tooth sites/patient, sample pooling, quantification by ELISA in the laboratory.

3.4.3.3 Visit 3: aMMP-8 measurement, initial periodontal treatment and drug dispensing

Within six weeks after visit 2, the next appointment was scheduled. Visit 3 consisted of two active treatment sessions, visits 3a and 3b, scheduled within 24 hours and a third follow-up visit, referred to as visit 3c, two weeks later.

Appointment 3a was initiated with aMMP-8 sampling from four previously designated sites. Subsequently, the treatment was conducted in the first and fourth quadrants (on the right side in the upper and lower jaw) and on the next day in the contralateral dentition. If feasible, the whole mechanical debridement was performed in a single visit. During the active phase, the routine supra- and subgingival debridement was performed. As in the pre-treatment phase, ultrasonic scalers or airscalers were used, followed by area-specific, sharp hand instruments (Gracey curettes # 5/6, 7/8, 11/12, 13/14, Deppeler SA, Switzerland) and furcation curettes (SQBL 1 P, SQMD 1 P, Hu-Friedy, USA), if applicable. Local infiltration anaesthesia (Ultracain® D-S/ Ultracain® D-S forte, Sanofi-Aventis Deutschland GmbH, Germany) was provided as and when required. The treatment continued until the biofilm and calculus remnants were not detectable anymore with a calculus detection explorer (EXD 11/12, Hu-Friedy, USA). Finally the treated sites were irrigated subgingivally with a 3% solution of hydrogen peroxide until the bleeding stopped. Polishing with a polishing paste and rotating rubber cups as well as topical application of fluoride gel similarly to the pre-treatment phase followed. After the completion of mechanical debridement at the second appointment, the patient received either an adjunctive antimicrobial therapy consisting of oral metronidazole 400 mg (Flagyl® film-coated tablets, Sanofi Aventis Deutschland GmbH, Germany) and amoxicillin 500 mg (Amoxicillin-ratiopharm® 500, Ratiopharm GmbH, Germany) or two kinds of placebo drugs (P-tablets white 8 mm Lichtenstein, Winthrop Arzneimittel GmbH, Germany). They had to be taken every 8 hours for seven days, one capsule from each bottle at a time. The prefabricated antibiotics and placebo drugs were blinded using gelatine capsules. Additionally, all patients were prescribed a 0.2% chlorhexidine-digluconate mouth rinse to be used twice daily for seven days (Chlorhexamed, John O. Butler - Sunstar Deutschland GmbH, Germany). If desired, a painkiller was recommended (Ibuprofen 400 mg, Ratiopharm GmbH, Germany).

14 days later, an additional appointment was scheduled and aMMP-8 sampling was carried out. The patients returned empty medication packages, residual study drugs and the medication diary and were explicitly asked about possible adverse events.

The sequence of procedures at individual teeth is presented in Figure 8.

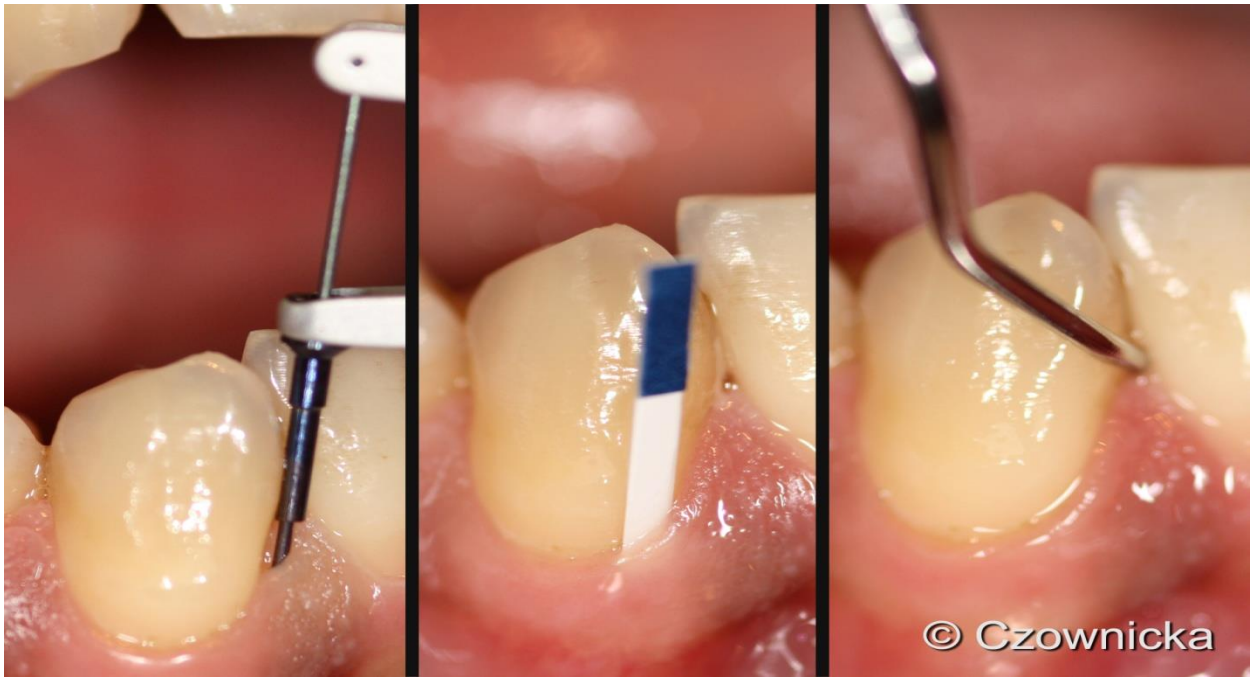

Fig. 8 Sequence of procedures: obtaining periodontal data with a Florida probe handpiece, collecting of aMMP-8 sample, mechanical treatment.

3.4.3.4 Visit 4: aMMP-8 measurement and re-evaluation

Two months (± 2 weeks) after visit 3, four aMMP-8 samples and four subgingival microbiological samples from sample teeth were collected, respectively. The questionnaires were completed and the carbon monoxide measurements in the exhaled air were performed. A clinical examination was performed, the Plaque Index was calculated and clinical measurements of RAL in duplicate (with a Florida disk probe® handpiece) as well as BOP, PD, Rec (standard Florida probe® handpiece) and furcation involvement (Nabers probe) were carried out.

3.4.3.5 Visit 5: aMMP-8 measurement, follow-up measurements and maintenance therapy

Three months (± 4 weeks) after visit 4, the patients were seen and four aMMP-8 samples and four subgingival samples from sample teeth were collected. The clinical inspection was performed, the Plaque Index was calculated and the clinical measurements of RAL in duplicate (with a Florida disk probe® handpiece) as well as BOP, PD, Rec (standard Florida probe® handpiece) were carried out. Depending on the

number of natural teeth and the extent of periodontal disease, a routine supra- and subgingival debridement was performed on the same visit or a separate appointment was scheduled within the shortest time possible. Mechanical treatment with ultrasonic scalers or airscalers was performed, followed by area specific hand instrumentation. Local anaesthesia was given on patient's demand before treatment. Polishing and fluoride gel application followed. Oral hygiene instructions were renewed according to patient's individual needs.

3.4.3.6 Visit 6: follow-up measurements and maintenance therapy

Three months (± 4 weeks) after visit 5, the patient was scheduled for a follow-up visit. A clinical inspection was performed, the Plaque Index was calculated and the clinical measurements of RAL in duplicate (with a Florida disk probe® handpiece) as well as BOP, PD, Rec (standard Florida probe® handpiece) were carried out. Additionally, questionnaires were completed. After completion of all measurements and depending on the number of natural teeth and the extent of periodontal disease, a routine supra- and subgingival debridement was performed on the same visit or during the separate appointment which was scheduled within the shortest time possible. Mechanical treatment with ultrasonic scalers or airscalers was performed, followed by area-specific hand instrumentation. Local anaesthesia was given on patient's demand before treatment. Polishing and fluoride gel application followed. Oral hygiene instructions were renewed according to patient's individual needs.

3.4.3.7 Visits 7-12: follow-up measurements and maintenance therapy

The aMMP-8 measurement as part of the sub-study came to an end at visit 6, but the main study and further sub-study measurement continued until visit 12, with patients treatment phase running out at the end of December 2011. Patients were further attended to by members of the ABPARO study centre in Berlin. After study closing visit, patients were offered subsequent treatment possibility within the scope of student courses at the Institute for Dental, Oral and Maxillary Medicine of Charité-Universitätsmedizin Berlin. Alternatively, the clinically relevant data were copied and forwarded to patient's general dental practitioner.

3.5 Statistical analysis

3.5.1 Examiner calibration

To achieve a continuity of patient care and reduce inter-examiner variability, one health provider conducted examination and treatment whenever possible. To additionally minimize intra- and inter-examiner differences, a calibration of the examiners involved was performed thrice over the course of the study (Grossi *et al*., 1996) Intra-examiner reproducibility was achieved at 95% level within ± 1mm.

3.5.2 Sample size

Sample size comprised of 34 subjects and was limited to the amount of patients who qualified for the main study in the study centre Berlin and who during the time of a first MPP-8 sampling had not reached the stage of an initial periodontal therapy. Three patients were excluded from statistical analysis as they discontinued their participation in the study before the second sampling; hence, final statistical analysis was performed for 31 subjects (see Figure 4).

3.5.3 Statistical analysis

Data bank of all main study patients with clinically measured values was converted with the Software FP 32 Data Downloader (Florida Probe Corporation, USA) into an Excel file (Excel 97, Microsoft, USA). The data of patients where aMMP-8 was measured were extracted and entered in separate Excel spread sheets. The Excel table was completed with the laboratory data and relevant data from Case Report Forms were added manually. The raw data were prepared for the statistical analysis which was performed with the Statistical Package for Social Science (SPSS, USA) with the support of the Institute for Biometry and Clinical Epidemiology (Charité Medical University, Germany). Descriptive statistics of the included data was given using contingency tables, line diagrams and box-and-whisker plots. Data were compared at the patient level (calculated as a mean value per patient per visit) or at the site level, where every measurement or collection point was considered as a separate value. The statistical analysis did not include possible differences between results achieved in patients who took antibiotics and those who did not, nor differences resulting from smoking habit. A non-parametric statistical test for matched pairs, Wilcoxon signed-rank test, was used for the comparison of particular values at different time points. A two-tailed p-value less than 0.05 was considered as significant. Ability of forecasting periodontal disease

progression was checked by constructing a receiver operating characteristic (ROC) curve and calculation of area under the curve (AUC). An asymptotic confidence interval of 95% was chosen. Clinically relevant changes of pocket depth and clinical attachment level were calculated separately by setting a level of clinical significance at 0.5 mm difference between the values. Deep pockets, i.e. a minimum of 5mm were clustered and analysed separately for disease progression. Non-parametric correlations between pocket depth and aMMP-8 concentration at the site level were analysed using Spearman's rank Correlation Coefficient, with p-value considered as significant if less than 0.05. A Mann-Whitney test was used to analyse a non-parametric correlation between the tested parameters and presence/absence of bleeding on probing at those sites, with a threshold of 0.05 considered as statistically significant.

4. Results

4.1 Study subjects

From 34 patients recruited into the study, one patient left the study at visit 2, before samples adequate for statistical analysis were collected and therefore the concentration of aMMP-8 was analysed for 33 patients. Additionally, the clinical data of 2 further patients was not evaluated statistically; one patient did not show up after visit 3a and in the case of one patient, the aMMP-8 sampling was not carried out on subsequent visits despite his further participation in the main study. Thus, 31 patients reached the final aMMP-8 sampling at visit 5 out of whom 27 subjects showed up for the final clinical examination at visit 6. The mean age of study patients at the time of signing the informed consent was 52 years (+/- 10 years, range between 33 and 73). With respect to the gender, the patient groups were equally divided, with 17 females (55%) and 14 males (45%). Concerning tobacco consumption, there were 17 smokers, out of whom 7 were found to be as moderate smokers with an average consumption of 1 packet of cigarettes per day and 14 were non-smokers.

4.2. Clinical and laboratory parameters

4.2.1 Clinical parameters at the patient level

4.2.1.1 Plaque Index

The plaque deposition during the observation period remained at a low and stable level of 11-15% of all tooth sites. As a result, the O'Leary's Plaque Index was at very low

levels at all the measurement points, with differences not reaching statistical significance between visits ($p > 0.05$) as presented in Table VII and VIII.

Table VII: Plaque Index (O'Leary *et al*., 1972) values calculated from the mean values of all sites of each patient at baseline, at visits 4, 5 and 6; value expressed as a fraction of 1, 1 = 100%

	Plaque Index			
	Plaque V2 Baseline	**Plaque V4 4 Months**	**Plaque V5 7 Months**	**Plaque V6 10 Months**
Number	31	31	30	27
Mean	**0.11**	**0.11**	**0.13**	**0.15**
Median	0.06	0.05	0.11	0.10
Standard Deviation	0.13	0.15	0.13	0.16
Minimum	0.00	0.00	0.00	0.00
Maximum	0.60	0.76	0.58	0.63
Percentile 25	0.03	0.02	0.05	0.04
50	0.06	0.05	0.11	0.10
75	0.15	0.17	0.18	0.24

Table VIII: Changes in the mean Plaque Index (O'Leary *et al*., 1972) values between visits and their level of significance (Wilcoxon signed-rank test); value expressed as a fraction of 1, 1 = 100%

	Plaque Index					
	V4-V2 0-4 months	**V5-V2 0-7 months**	**V6-V2 0-10 months**	**V5-V4 4-7 months**	**V6-V4 4-10 months**	**V6-V5 7-10 months**
Differences between visits	0.00	0.03	0.05	0.03	0.05	0.02
p-value	0.766	0.062	0.275	0.089	0.219	0.486

Baseline mean Plaque Index values and their changes throughout the study period are illustrated in Figure 9.

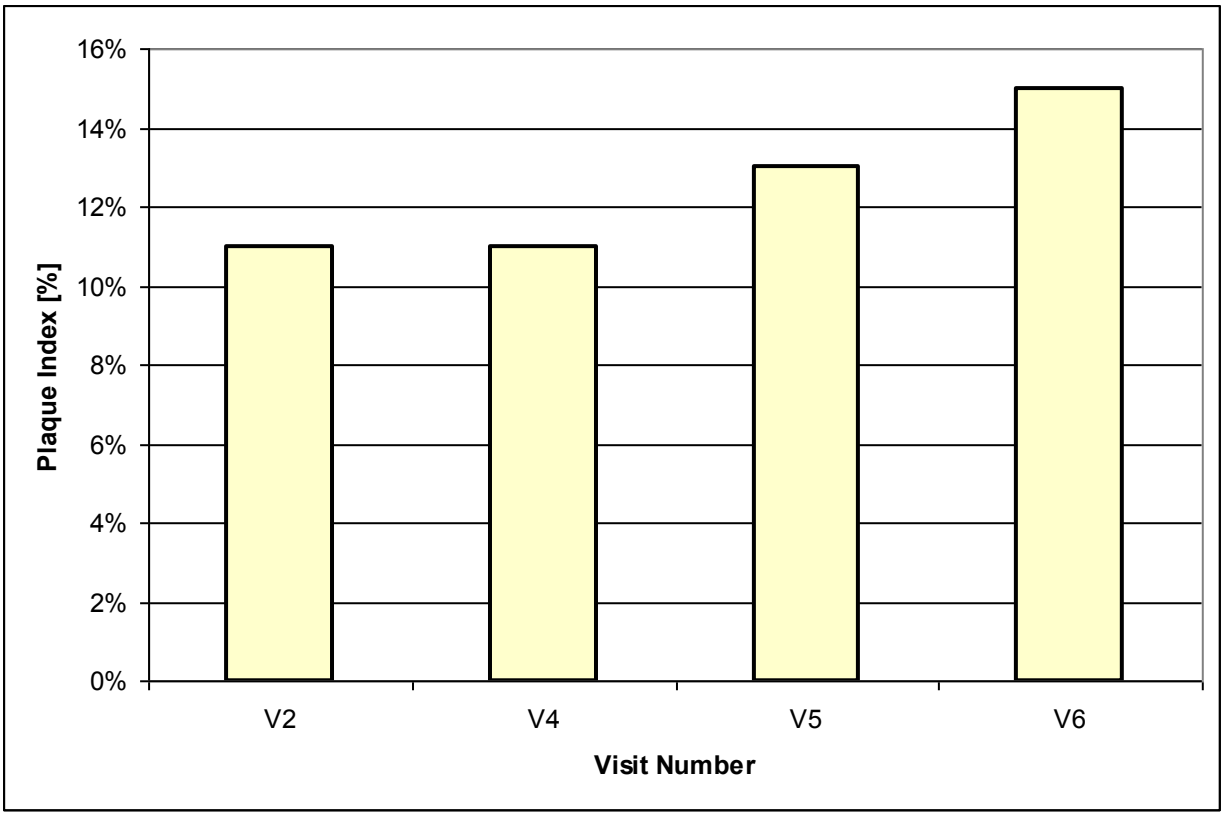

Fig. 9 Mean Plaque Index values (O'Leary *et al*., 1972) calculated from the mean values of all sites of each patient measured at visits 2 (31 patients), 4 (31 patients), 5 (30 patients), and 6 (27 patients).

4.2.1.2 Periodontal pocket depth

After the first 4 months, there was a considerable improvement in median pocket depths, a mean shallowing of 0.5 mm was achieved ($p < 0.001$) as presented in Tables IX and X. Similarly, median periodontal pocket depth was reduced significantly between 7^{th} and 10^{th} month from the baseline measurements ($p = 0.014$).

Table IX: Pocket depth (PD) values calculated from the mean values of all sites of each patient at baseline, visits 4, 5 and 6; expressed in mm

	Pocket Depth			
	PD V2 Baseline	**PD V4 4 Months**	**PD V5 7 Months**	**PD V6 10 Months**
Number	31	31	30	27
Mean	3.49	3.86	2.84	2.71
Median	**3.27**	**2.78**	**2.80**	**2.62**
Standard Deviation	0.76	0.65	0.59	0.66
Minimum	2.19	1.65	1.65	1.78
Maximum	5.67	4.73	4.66	5.18
Percentile 25	3.02	2.49	2.50	2.37
50	3.27	2.78	2.80	2.62
75	3.86	3.30	3.17	2.93

Table X: Changes in the median pocket depth (PD) values, expressed in mm, between visits and their level of significance (Wilcoxon signed-rank test)

	Pocket Depth					
	V4-V2	**V5-V2**	**V6-V2**	**V5-V4**	**V6-V4**	**V6-V5**
	0-4 months	**0-7 months**	**0-10 months**	**4-7 months**	**4-10 months**	**7-10 months**
Differences between visits	-0.49	-0.47	-0.65	0.02	-0.16	-0.18
p-value	0.000	0.000	0.000	0.465	0.061	0.014

Changes in median pocket depths are illustrated in Figure 10. The initial, highest PD value at visit 2 was never reached again in further measurements during the study. There is a tendency to continuous PD reduction with time, with no statistically significant increase between visits 4 and 5.

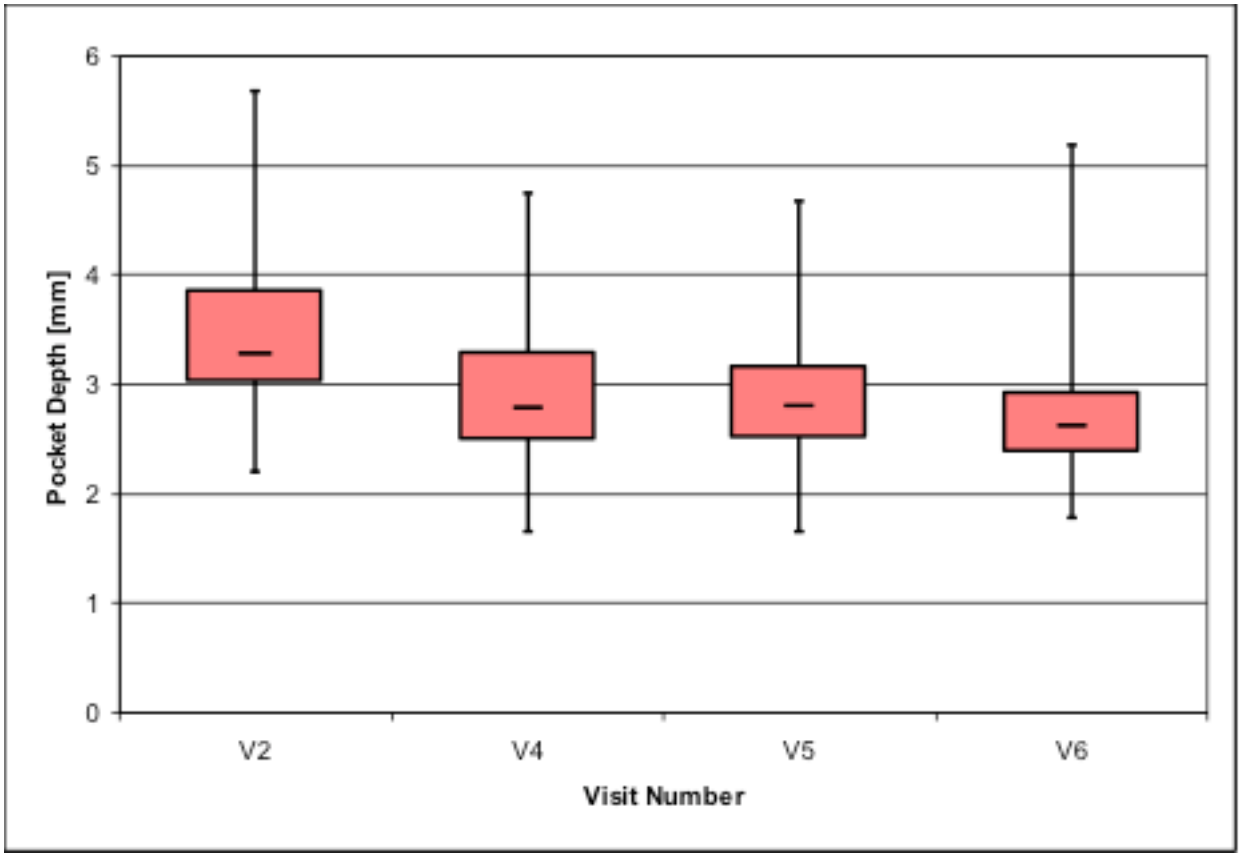

Fig. 10 Box-and-whisker plot showing median, quartile and extreme values of pocket depth, calculated from the mean values of all sites of each patient measured at visits 2 (31 patients), 4 (31 patients), 5 (30 patients) and 6 (27 patients).

4.2.1.3 Clinical attachment level

Within the first 4 months, there was a significant decrease in median clinical attachment level (-0.68 mm, $p < 0.001$, Tables XI and XII), in other words a “gain” of clinical attachment. The changes between month 4 and month 7 did not reach a statistical

significance level (p = 0.428). Between 7th and 10th months a significant decrease in CAL could be observed again (p = 0,007).

Table XI: Clinical attachment level (CAL) values calculated from the mean values of all sites of each patient at baseline, visit 4, 5 and 6; expressed in mm

	Clinical Attachment Level			
	CAL V2 Baseline	**CAL V4 4 Months**	**CAL V5 7 Months**	**CAL V6 10 Months**
Number	31	31	30	27
Mean	4.36	3.98	4.06	3.85
Median	**4.25**	**3.57**	**3.91**	**3.68**
Standard Deviation	0.93	1.05	1.09	1.05
Minimum	3.01	2.39	2.45	2.04
Maximum	6.73	6.64	6.88	6.65
Percentile 25	3.64	3.25	3.28	3.12
50	4.25	3.57	3.91	3.68
75	5.05	4.97	4.81	4.47

Table XII: Changes in the median clinical attachment level (CAL) values, expressed in mm, between visits and its level of significance (Wilcoxon signed-rank test)

	Clinical Attachment Level					
	V4-V2 0-4 months	**V5-V2 0-7 months**	**V6-V2 0-10 months**	**V5-V4 4-7 months**	**V6-V4 4-10 months**	**V6-V5 7-10 months**
Differences between visits	-0.68	-0.34	-0.57	0.33	0.11	-0.22
p-value	0.000	0.001	0.000	0.428	0.156	0.007

Changes in clinical attachment level values are illustrated in Figure 11.

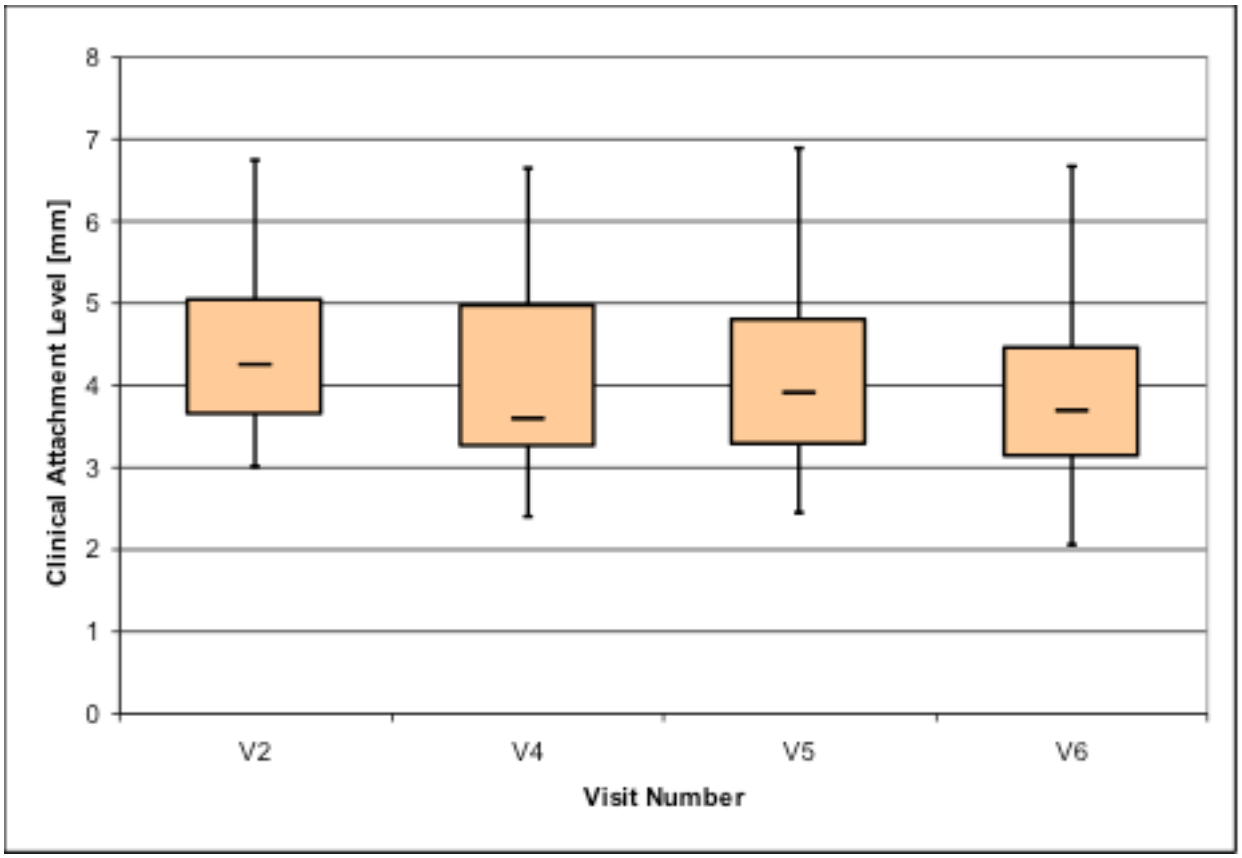

Fig. 11 Box-and-whisker plot showing median, quartile and extreme values of clinical attachment level calculated from the mean values of all sites of each patient measured at visits 2 (31 patients), 4 (31 patients), 5 (30 patients) and 6 (27 patients).

4.2.1.4 Bleeding on probing

During basement measurements every fourth tooth site showed bleeding during probing (Table XIII). After 4 months, the whole bleeding on probing score decreased significantly by 12% ($p < 0.001$, Table XIV). Between 4th and 7th month after the baseline, there was a slight increase of the parameter from 14 to 18% ($p = 0{,}003$). There was no statistically significant change of BOP values between visits 5 and 6 ($p = 0.525$). Graphically the changes of mean bleeding on probing per patient are depicted in Figure 12.

Table XIII: Bleeding on probing (BOP) values calculated from the mean values of all sites of each patient at baseline, visits 4, 5 and 6. Value expressed as a fraction of 1, 1 = 100%

	Bleeding on Probing			
	BOP V2 Baseline	**BOP V4 4 Months**	**BOP V5 7 Months**	**BOP V6 10 Months**
Number	31	31	30	27
Mean	**0.26**	**0.14**	**0.18**	**0.17**
Median	0.26	0.12	0.14	0.14
Standard Deviation	0.11	0.08	0.11	0.10

Minimum	0.10	0.04	0.02	0.01
Maximum	0.53	0.42	0.48	0.35
Percentile 25	0.17	0.09	0.10	0.09
50	0.26	0.12	0.14	0.14
75	0.33	0.17	0.23	0.21

Table XIV: Changes in the mean Bleeding on probing (BOP) values between visits and their level of significance (Wilcoxon signed-rank test). Value expressed as a fraction of 1, 1 = 100%

	Bleeding on Probing					
	V4-V2	**V5-V2**	**V6-V2**	**V5-V4**	**V6-V4**	**V6-V5**
	0-4 months	**0-7 months**	**0-10 months**	**4-7 months**	**4-10 months**	**7-10 months**
Differences between visits	-0.12	-0.09	-0.10	0.04	0.03	-0.01
p-value	0.000	0.001	0.000	0.003	0.28	0.525

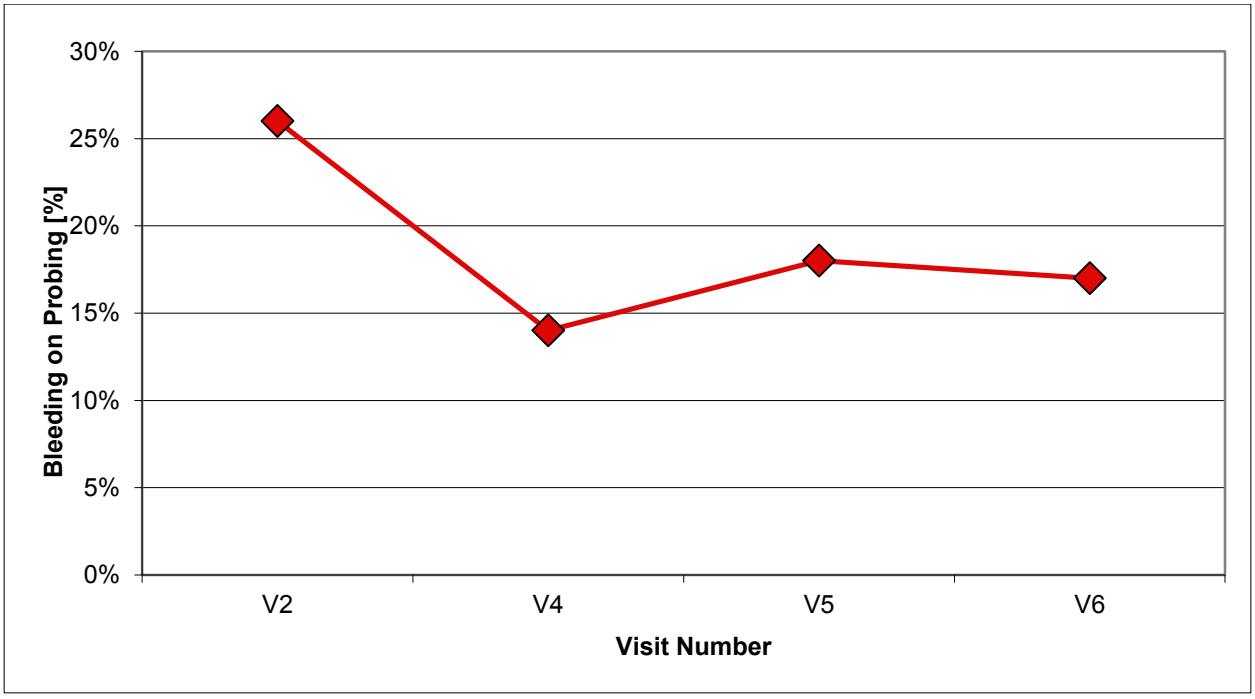

Fig. 12 Mean bleeding on probing values calculated from the mean values of all sites of each patient measured at visits 2 (31 patients), 4 (31 patients), 5 (30 patients), and 6 (27 patients).

4.2.2 Active matrix metalloproteinase 8 levels

The most pronounced change in the level of active MMP-8 took place between the first sampling, on the day of the active treatment at visit 3a, and at the control visit 3c two weeks later. Median values decreased statistically significantly by half from 5 ng/mL to 2.48 ng/mL ($p < 0.001$, Table XV and XVI). Changes between visits 3c and 4 failed to reach statistical significance ($p = 0.393$). Further increase of median enzyme level can be observed between visits 4 and 5, however, the results narrowly missed the threshold

of statistical significance (p = 0.054). Between the initial treatment appointment, visit 3a and the first clinical re-evaluation at visit 4, a markedly significant decrease of median aMMP-8 level was observed (p = 0.001). The lowest values were measured at visit 3c, 2 weeks after active therapy, from then on the values rose gradually up to final measurement at visit 5 (p = 0.042), however, they never reached the initial high levels of the pre-treatment status. At visit 3c, the distribution of aMMP-8 concentrations was more consistent, with the narrowest interquartile distance (IQ: 1.45, 5.2) (Figure 13). With only one active treatment between initial aMMP-8 measurement at visit 3a and final measurement at visit 5 there was a decline in median aMMP-8 levels within this period of 5.5 months, the statistical significance level had not been reached yet (p = 0.06).

Table XV: Active matrix metalloproteinase-8 (aMMP-8) values calculated from the values of all sites at baseline, visits 4, 5 and 6; expressed in ng/mL

	active Matrix Metalloproteinase-8			
	aMMP-8 V3a 1.5 months	**aMMP-8 V3c 2 Months**	**aMMP-8 V4 4 Months**	**aMMP-8 V5 7 Months**
Number	132	124	120	120
Mean	12.38	5.09	5.82	8.07
Median	**5.00**	**2.48**	**2.82**	**3.65**
Standard Deviation	18.33	7.13	9.40	13.13
Minimum	0.59	0.25	0.50	0.70
Maximum	81.87	38.54	69.81	76.10
Percentile 25	1.94	1.45	1.63	1.81
50	5.00	2.48	2.82	3.65
75	12.20	5.20	6.52	8.11

Table XVI: Changes in the median Active matrix metalloproteinase-8 (aMMP-8) values between visits and their levels of significance (Wilcoxon signed-rank test). Values expressed in ng/mL

	active Matrix Metalloproteinase-8					
	V3c-V3a 1.5-2 months	**V4-V3a 1.5-4 months**	**V5-V3a 1.5-7 months**	**V4-V3c 2-4 months**	**V5-V3c 2-7 months**	**V5-V4 4-7 months**
Differences between visits	-2.52	-2.18	-1.35	0.34	1.17	0.83
p-value	0.000	0.001	0.06	0.393	0.042	0.054

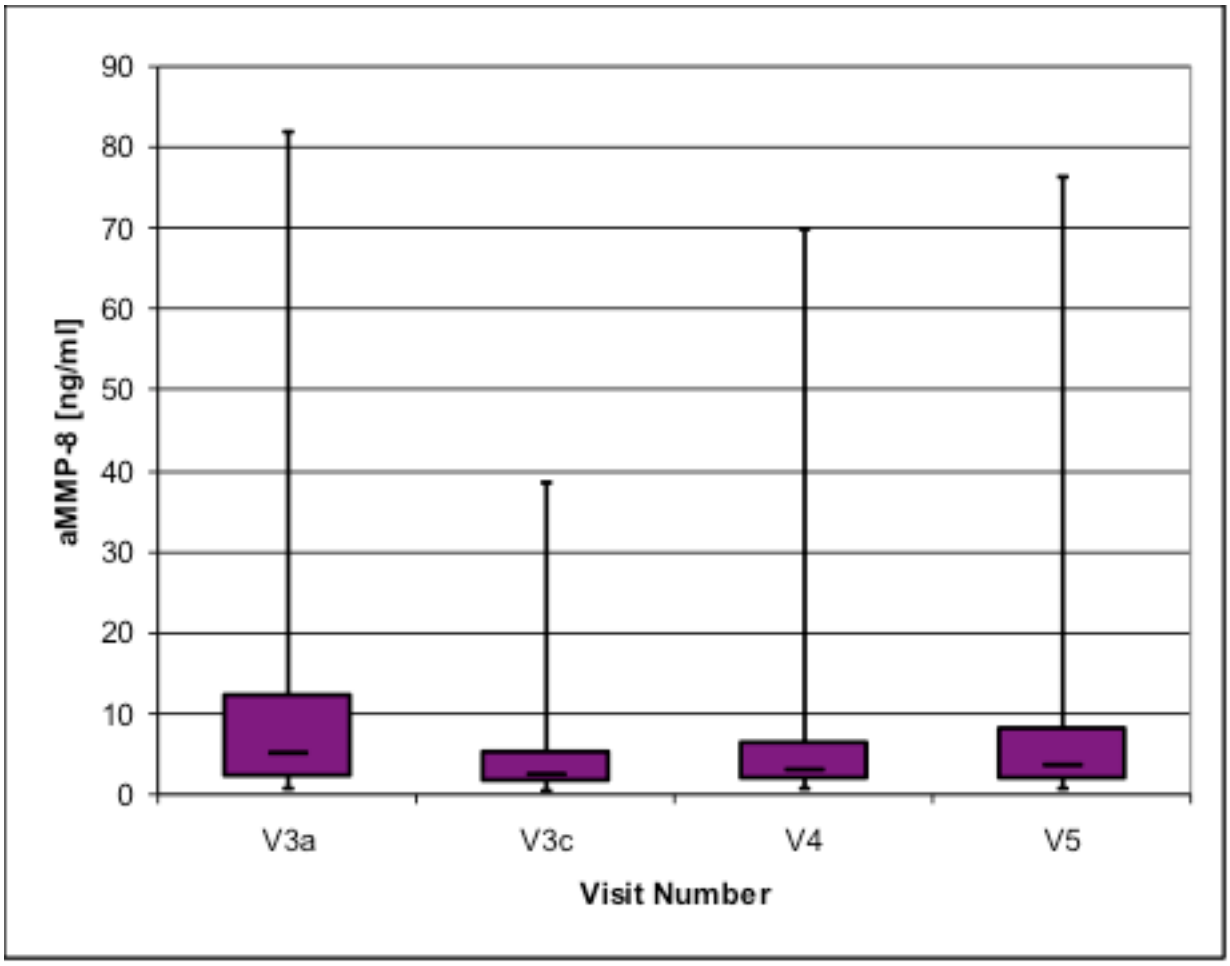

Fig. 13 Box-and-whisker plot showing median, quartile and extreme values of active matrix metalloproteinase-8 calculated from four sampling sites per patient at consecutive measurement visits 3a (33 patients), 3c (31 patients), 4 (30 patients) and 5 (30 patients).

4.2.3 Correlations between pocket depth and aMMP-8 at the site level

5.2.3.1 Correlation between pocket depth at visit 2 and aMMP-8 at visit 3a

There is a positive correlation between pocket depth measured at visit 2 at the site level and concentration of aMMP-8 sampled at visit 3a from the same sites (Spearman's rho = 0.18, p = 0.045, Table XVII).

Table XVII: Correlation between pocket depth of aMMP-8 sampling sites at visit 2 and concentration of aMMP-8 sampled at visit 3a

	PD V2	**aMMP-8 V3a**
Spearman's rho Correlation Coefficient	1	0.18
Level of Significance	0.045	
Number of Test Sites	124	

4.2.3.2 Correlation between pocket depth at visit 4 and aMMP-8 at visit 4

There is no correlation between pocket depths measured at visit 4 at the site level and concentration of aMMP-8 sampled at visit 4 from the same sites, due to lack of statistical significance (p > 0.05, Table XVIII).

Table XVIII: Correlation between pocket depth of aMMP-8 sampling sites at visit 4 and concentration of aMMP-8 sampled at visit 4

	PD V4	**aMMP-8 V4**
Spearman's rho Correlation Coefficient	1	0.14
Level of Significance	0.124	
Number of Test Sites	120[1]	

4.2.3.3 Correlation between pocket depth at visit 5 and aMMP-8 at visit 5

There is no correlation between pocket depth measured at visit 5 at the site level and concentration of aMMP-8 sampled at visit 5 from the same sites, due to lack of statistical significance (p > 0,05, Table XIX).

Table XIX: Correlation between pocket depth of aMMP-8 sampling sites at visit 5 and concentration of aMMP-8 sampled at visit 5

	PD V5	**aMMP-8 V5**
Spearman's rho Correlation Coefficient	1	0.16
Level of Significance	0.086	
Number of Test Sites	116	

4.2.4 Clinical and laboratory parameters depending on bleeding on probing

4.2.4.1 Pocket depth at visit 2

During visit 2, out of 124 sites, from which the enzyme aMMP-8 was sampled, 71 sites showed no bleeding on probing and 53 sites showed a positive result. Median pocket depth was identical in both groups, namely 5.4 mm. Differences were present in standard deviation and extreme values, nevertheless the differences did not reach the statistical significance (p > 0.05, Table XX).

[1] Number of sampled sites differs between visits, due to patient drop-outs or samples not being available for analysis

Table XX: Pocket depth values from the aMMP-8 sampling sites depending on the presence or absence of bleeding on probing at baseline. 0 = negative BOP, 1 = positive BOP, PD expressed in mm

	BOP V2 = 0	**BOP V2 = 1**
Number of Sites	71	53
Mean PD V2	5.21	5.51
Median PD V2	**5.40**	**5.40**
Standard Deviation	1.64	2.10
Minimum PD V2	1.60	1.60
Maximum PD V2	10.20	12.00
Level of Significance	0.468	

4.2.4.2 aMMP-8 concentration at visit 3a

Median aMMP-8 concentration measured at visit 3a was 3.09 ng/mL in the GCF from pockets, where no bleeding was detected during clinical data collection at visit 2 and 6.89 ng/mL if bleeding was present. Standard deviation was smaller in the group with negative BOP, but the extreme values, minimal and maximal, were in that group further distributed. Differences between both groups were statistically significant (p = 0.04, Table XXI).

Table XXI: Active matrix metalloproteinase-8 values from the aMMP-8 sampling sites at visit 3a depending on the presence or absence of bleeding on probing at visit 2. 0 = negative BOP, 1 = positive BOP, MMP-8 expressed in ng/mL

	BOP V2 = 0	**BOP V2 = 1**
Number of Sites	71	53
Mean MMP-8 V3a	9.73	12.91
Median MMP-8 V3a	**3.09**	**6.89**
Standard Deviation	15.55	18.09
Minimum MMP-8 V3a	0.59	0.63
Maximum MMP-8 V3a	81.87	77.78
Level of Significance	0.040	

4.2.4.3 Pocket depth at visit 4

Out of 124 sites, from which the enzyme aMMP-8 was sampled, 94 sites showed no bleeding on probing, whereas the bleeding was present in 30 sites during data collection at visit 4. Median pocket depth was 0.5 mm smaller in the BOP-negative

group (4 mm versus 4.5 mm, Table XXII). The differences however did not reach statistical significance (p > 0.05. Table XXII).

Table XXII: Pocket depth values from the aMMP-8 sampling sites at visit 4 depending on the presence or absence of bleeding on probing at visit 4. 0 = negative BOP, 1 = positive BOP, PD expressed in mm

	BOP V4 = 0	**BOP V4 = 1**
Number of Sites	94	30
Mean PD V4	3.99	4.39
Median PD V4	**4.00**	**4.50**
Standard Deviation	1.57	1.99
Minimum PD V4	0.80	0.80
Maximum PD V4	9.80	8.00
Level of Significance	0.202	

4.2.4.4 aMMP-8 concentration at visit 4

During visit 4, out of 120 sites, from which the enzyme aMMP-8 was sampled, 93 sites showed no bleeding on probing and 27 sites showed a positive result. Median aMMP-8 concentration was 2.4 ng/mL in the pockets where no bleeding was detected and 6.1 ng/mL if the bleeding was present. Standard deviation was smaller in the group with negative BOP and the range between minimal and maximal values was also narrower. The differences between the two groups were statistically significant (p = 0.001, Table XXIII).

Table XXIII: Active matrix metalloproteinase-8 values from the aMMP-8 sampling sites at visit 4 depending on the presence or absence of bleeding on probing at visit 4. 0 = negative BOP, 1 = positive BOP, PD expressed in mm

	BOP V4 = 0	**BOP V4 = 1**
Number of Sites	93	27
Mean MMP-8 V4	3.92	12.35
Median MMP-8 V4	**2.40**	**6.10**
Standard Deviation	4.18	16.90
Minimum MMP-8 V4	0.50	0.96
Maximum MMP-8 V4	28.84	69.81
Level of Significance	0.001	

5.2.4.5 Pocket depth at visit 5

Out of 120 sites, from which the enzyme aMMP-8 was sampled, 88 sites showed no bleeding on probing, whereas bleeding was present at 32 sites during data collection at visit 5. Median pocket depth of BOP-negative sites stayed on the same level as during visit 4 (4 mm), however, median PD in the BOP-positive sites decreased from 4.5 to 3.5 mm and was smaller than in the group with a negative BOP (Table XXIV). The differences however did not reach statistical significance ($p > 0.05$).

Table XXIV: Pocket depth values from the aMMP-8 sampling sites at visit 5 depending on the presence or absence of bleeding on probing at visit 5. 0 = negative BOP, 1 = positive BOP, PD expressed in mm

	BOP V5 = 0	**BOP V5 = 1**
Number of Sites	88	32
Mean PD V5	4.07	4.37
Median PD V5	**4.00**	**3.50**
Standard Deviation	1.69	1.80
Minimum PD V5	1.00	1.60
Maximum PD V5	10.20	7.60
Level of Significance	0.527	

4.2.4.6 aMMP-8 concentration at visit 5

During visit 5, out of 116 sites, from which the enzyme aMMP-8 was sampled, 84 sites showed no bleeding on probing and 32 sites showed a positive result. Median aMMP-8 concentration was 3.68 ng/mL in the pockets where no bleeding was detected and 4 ng/mL, if the bleeding was present. However, the differences between the groups did not reach the statistical significance ($p > 0.05$, Table XXV).

Table XXV: Active matrix metalloproteinase-8 values from the aMMP-8 sampling sites at visit 5 depending on the presence or absence of bleeding on probing at visit 5. 0 = negative BOP, 1 = positive BOP, MMP--8 expressed in ng/mL

	BOP V5 = 0	**BOP V5 = 1**
Number of Sites	84	32
Mean MMP-8 V5	7.84	9.55
Median MMP-8 V5	**3.68**	**4.01**
Standard Deviation	13.08	13.98
Minimum MMP-8 V5	0.73	1.09
Maximum MMP-8 V5	76.10	55.69
Level of Significance	0.684	

4.2.5 Prediction of activity

4.2.5.1 Prediction of disease activity using aMMP-8 collected at visit 3a

Based on mean probing depth, 2 patients suffered disease progression between visit 2 and visit 4. In 31 patients the mean probing depth decreased between those visits. Similar results were obtained when the pooled data from 4 sites per patient were analysed. When only pockets of a minimum of 5 mm in depth were considered, 1 patient was classified as a patient with disease progression and in 32 patients the mean deep pocket depths decreased at visit 4. In none of the cases could the level of aMMP-8 measured during visit 3a predict the changes of pocket depths between visit 2 and visit 4, as the level of statistical significance was not achieved ($p > 0.05$, Table XXVI).

Based on mean clinical attachment level, in the case of 4 subjects, the disease progression was determined, whereas 29 patients showed improvement of their mean clinical attachment levels. Taking into account only 4 sites per patient, from which the enzyme was sampled, the same results were obtained. However, clinically relevant loss of CAL between visit 2 and visit 4, i.e. of a minimum of 0.5 mm, was observed only in 1 patient. None of the changes could be predicted by the level of aMMP-8 sampled at visit 3a due to lack of statistical significance ($p > 0.05$, Table XXVI).

Table XXVI: Disease activity between visit 2 and visit 4 defined by pocket deepening or loss of clinical attachment level, and the ability of aMMP-8 concentration measured at visit 3a to predict respective changes

MMP-8V3aPool	Disease Activity Number of cases		AUC	CI	p-value
	Progression +	Progression -			
meanPDV4 > meanPDV2	2	31	0.45	(0.00; 1.00)	0.821
PDV4pool > PDV2pool	2	31	0.47	(0.00; 1.00)	0.880
PD5mmV4 > PD5mmV2	1	32	0.84	(0.72; 0.97)	0.248
meanCALV4 > meanCALV2	4	29	0.56	(0.22; 0.90)	0.699
CALV4pool > CALV2pool	4	29	0.38	(0.14; 0.63)	0.456
CALV4pool > (CALV2pool + 0.5)	1	32	0.56	(0.39; 0.73)	0.834

AUC = area under the curve; CI = confidence interval

4.2.5.2 Prediction of disease activity using aMMP-8 collected at visit 3c

Concentration of aMMP-8 measured at visit 3c could not predict the disease progression between visit 2 and visit 4, neither regarding the pocket depth nor the clinical attachment level, as the p-value did not reach the level of statistical significance ($p > 0.05$, Table XXVII).

Table XXVII: Disease activity between visit 2 and visit 4 defined by pocket deepening or loss of clinical attachment level, and the ability of aMMP-8 concentration measured at visit 3c to predict respective changes

MMP-8V3cPool	Disease Activity Number of cases		AUC	CI	p-value
	Progression +	Progression -			
meanPDV4 > meanPDV2	2	29	0.59	(0.09; 1.00)	0.688
PDV4pool > PDV2pool	2	29	0.80	(0.66; 0.95)	0.159
PD5mm V4 > PD5mmV2	1	30	0.93	(0.84; 1.00)	0.146
meanCALV4 > meanCALV2	4	27	0.57	(0.23 ;0.91)	0.680
CALV4pool > CALV2pool	4	27	0.74	(0.44; 1.00)	0.133
CALV4pool > (CALV2pool + 0.5)	1	30	0.93	(0.84; 1.00)	0.146

4.2.5.3 Prediction of disease activity using aMMP-8 collected at visit 4

Analysed at the subject level, in the case of 11 patients, the disease progressed between visits 4 and 5. When only particular sites, from which the enzyme samples were collected, were taken into consideration, 15 patients suffered disease progression. Clinically significant increase of pocket depth in those particular sites, i.e. of minimum 0.5 mm, occurred in 6 out of 30 patients. Taking into consideration only deep pockets, i.e. of minimum 5mm, 11 patients suffered disease progression. In none of the cases could the level of aMMP-8 measured during visit 4 predict the changes of pocket depth between visit 4 and visit 5, as the level of statistical significance was not achieved ($p > 0.05$, Table XXVIII).

Based on mean clinical attachment level, in the case of 14 subjects, disease progression occurred between visits 4 and 5, whereas 16 patients remained stable or gained the CAL. Taking into account only 4 sites per patient, from which the enzyme was sampled, 11 patients showed aggravation of the clinical attachment level. Clinically

relevant loss of CAL between visit 4 and visit 5, i.e. a minimum of 0.5 mm, was observed in 8 patients. None of the changes could be predicted by the level of aMMP-8 sampled at visit 4 due to lack of statistical significance ($p > 0.05$, Table XXVIII).

Table XXVIII: Disease activity between visit 4 and visit 5 defined by pocket deepening or loss of clinical attachment level, and the ability of aMMP-8 concentration measured at visit 4 to predict respective changes

MMP-8V4Pool	Disease Activity Number of cases		AUC	CI	p-value
	Progression +	Progression -			
MeanPDV5 > meanPDV4	11	19	0.43	(0.22; 0.65)	0.547
PDV5pool > PDV4pool	15	15	0.49	(0.27; 0.71)	0.901
PDV5pool > (PDV4pool + 0.5)	6	24	0.32	(0.06; 0.58)	0.186
PD5mmV5 > PD5mmV4	11	19	0.34	(0.14; 0.54)	0.149
MeanCALV5 > meanCALV4	14	16	0.58	(0.37; 0.79)	0.454
CALV5pool > CALV4pool	11	19	0.45	(0.21; 0.69)	0.651
CALV5pool > (CALV4pool + 0.5)	8	22	0.31	(0.07; 0.55)	0.116

4.2.3.4 Prediction of disease activity using aMMP-8 collected at visit 5

Based on mean probing depth, 8 patients suffered disease progression between visit 5 and visit 6, whereas in 22 patients the mean probing depth decreased between those visits. Similar results were obtained when the pooled data from 4 sites per patient were analysed; 9 patients experienced disease progression. However, clinically relevant deepening of pocket depth in those sites, i.e. a minimum of 0.5 mm, was observed only in 4 patients. When just pockets of a minimum of 5 mm were considered, 10 patients were classified as patients with disease progression and in 20 patients the deep mean pocket depths were smaller at visit 6. In none of the cases could the level of aMMP-8 measured during visit 5 predict the changes of pocket depths between visit 5 and visit 6, as the level of statistical significance was not achieved ($p > 0.05$, Table XXIX).

Based on mean clinical attachment level, in the case of 5 subjects the disease progression was determined, whereas 25 patients showed improvement of their mean clinical attachment levels. Taking into account only 4 sites per patient, from which the enzyme was sampled, 9 patients experienced deterioration of their periodontal condition

between visit 5 and visit 6. In 6 patients out of those 9, the change was clinically relevant, with a minimum of 0.5 mm. None of the changes could be predicted by the level of aMMP-8 sampled at visit 5 due to lack of statistical significance ($p > 0.05$, Table XXIX).

Table XXIX: Disease activity between visit 5 and visit 6 defined by pocket deepening or loss of clinical attachment level, and the ability of aMMP-8 concentration measured at visit 5 to predict respective changes

MMP-8V5Pool	Disease Activity Number of cases		AUC	CI	p-value
	Progression +	Progression -			
meanPDV6 > meanPDV5	8	22	0.30	(0.08; 0.53)	0.101
PDV6pool > PDV5pool	9	21	0.41	(0.17; 0.66)	0.455
PDV6pool > (PDV5pool + 0.5)	4	26	0.23	(0.07; 0.39)	0.088
PD5mmV6 > PD5mmV5	10	20	0.38	(0.17; 0.60)	0.301
meanCALV6 > meanCALV5	5	25	0.37	(0.11; 0.64)	0.373
CALV6pool > CALV5pool	9	21	0.39	(0.15; 0.64)	0.365
CALV6pool > (CALV5pool + 0.5)	6	24	0.44	(0.18; 0.71)	0.678

4.3 Summary of the results

The mean plaque level of the patients expressed by the O'Leary's Plaque Index remained at the same level on all the measurement visits. The median PD and median CAL of the patients were the highest at the baseline visit and decreased during the course of the study except between visits 4 and 5, where they did not change. Mean BOP was the highest at the baseline visit, it decreased significantly following the initial treatment, increased at visit 4 and remained at that level during visit 5. However, the initial, highest values were never reached again. The mean aMMP-8 level calculated from four sites per patient were highest at the first sampling visit, dropped by half two weeks after non-surgical periodontal treatment was performed and remained stable up to the last measurement visit.

Initial aMMP-8 levels and initial PD measured at the sampling sites showed correlation, however this interdependence was not statistically significant at other time-points. In none of the time-points did the presence of BOP correlate with PD due to lack of

statistical significance. Sites with positive BOP had higher median aMMP-8 levels at those sites except for visit 5, where the difference was not statistically significant.
Regardless of the definition of disease progression, levels of aMMP-8 sampled from four sites per patient did not predict disease progression at the patient level found at the consecutive visit ($p>0.05$).

5. Discussion

Periodontitis is the most common destructive condition of tooth-supporting structures in man. Untreated, it leads to progressing tissue breakdown and possibly, to subsequent tooth loss (Page and Kornman, 1997). Currently, clinical diagnostic parameters, such as pocket probing depth, clinical attachment level, and bleeding on probing are limited to estimating the irreversible, previous tissue destruction. Standard diagnostic methods fail nonetheless to detect the onset of the inflammation, provide no real-time assessment of disease status and have very limited prognostic value to identify patients and sites susceptible to future disease advancement (Giannobile *et al.*, 2009). Also radiologically recognizable signs of bone loss by calcium diminution can be retarded by 6 to 9 months in relation to the initiation of destructive processes in the tissues (Fine *et al.*, 2009). The aim of this study was to determine if the levels of aMMP-8 in GCF can predict periodontitis progression between a given time point and subsequent visits during supportive periodontal therapy at the patient level. A secondary objective was to determine if the levels of aMMP-8 correlate with clinical parameters in the cohort of periodontally involved individuals at the site level. Oral fluids are easily collected sources of biomarkers of oral and systemic diseases. A biomarker that precedes radiological and clinical evidence of tissue breakdown is highly desirable for early diagnostics in a subclinical phase. MMP-8 is the key collagenase secreted by host cells recruited during periodontal inflammation that mediates connective tissue and bone matrix degradation (Sorsa *et al*., 2006, Yucel-Lindberg and Bage, 2013). Validation of a biomarker requires the verification of its capability to differentiate the status of a disease accurately, its correlation with disease activity and progression and finally it's suitability for rapid point-of-care (POC) chair-side diagnostics required by the dental professionals. Previously published studies reported on the ability of MMP-8 to differentiate the disease status between the healthy and the periodontitis-affected sites based on the enzyme level in the GCF (Prescher *et al.*, 2007). Increased GCF MMP-8 levels were associated with increased odds (OR= 1.50) of subsequent periodontal

attachment loss (Reinhardt *et al.*, 2010). When multiple salivary biomarkers, including MMP-8, which was ranked with the highest importance, were combined with the microbial biofilm, the capacity to identify patient periodontal status increased markedly (Ramseier *et al.*, 2009). In our longitudinal study, where 4 sites in 31 patients were analysed statistically for the GCF level of aMMP-8, we obtained inconsistent results. We found a positive correlation between aMMP-8 level and PD during the initial measurement, with no correlation on subsequent maintenance visits. Higher levels of aMMP-8 were associated with present BOP in those sites during the initial and following visit, however, there was no correlation during the last sampling visit. In testing the predictive value of pooled samples at the patient level, the aMMP-8 could not predict disease activity at any of the time-points and regardless of definition of disease progression ($p>0.05$). Similarly ambiguous data can be found in the literature. Kinney *et al.,* who evaluated salivary biomarkers in periodontal disease progression, reported significant reductions in salivary MMP-8 concentration 8-12 months after SRP in the moderate/severe periodontitis group: however, these differences were not statistically significant in the mild periodontitis or in the gingivitis group (Kinney *et al.*, 2011). In the study of Ozcaka *et al.* who investigated the influence of smoking on serum concentrations of matrix metalloproteinase-8 they found differences in the healthy controls but no significant difference in MMP-8 concentrations or MMP-8/TIMP-1 ratio between chronic periodontitis group and periodontally healthy group (Ozcaka *et al.*, 2011). Mäntylä *et al.* who tested the efficacy of the MMP-8-specific chair-side dip-stick test for GCF in patients with chronic periodontitis found no difference between MMP-8 concentrations in progressing versus stable sites in smokers or in non-smokers. However, sites with persistently elevated MMP-8 concentrations during the maintenance phase indicated sites with poor response to treatment, that is no statistically significant improvement of PD or CAL after SRP (Mäntylä et al., 2006). But it is difficult to compare directly the results of different studies due to the large heterogeneity of chosen materials and methods. Various enzyme sources were used, such as GCF, whole saliva, or mouth rinse. Miscellaneous definitions of periodontal disease and progression were adopted, including subjective definitions, different clinical parameters and different cut-off levels were used. Various laboratory and chair-side methods were implemented to compute the MMP-8 level, with separate norm scales and units. Only the active form of MMP-8 was assessed or no differentiation was made

between active and latent form of the enzyme. Finally, the MMP-8 was tested alone, or in combination with other biochemical components or putative periodontal pathogens.

5.1 Study subjects

In the present study, 34 patients were recruited to validate the diagnostic capacity of aMMP-8. Sample size was determined by the number of individuals who were included in the main ABPARO study and was limited to those who at the time-point of the first required MMP-8 sampling had not undergo the initial mechanical therapy yet. Automatically, the inclusion criteria for the study were the same as those for the main study. As a result, a heterogeneous group of patients diagnosed both with the aggressive and with the chronic form of periodontitis formed the study sample, and the minors were excluded from the study. In other study designs, the form of periodontitis was usually part of the inclusion criteria, as shown in Table XXX. Mainly chronic periodontitis and gingivitis in adults were analysed, as they are the most prevalent forms of periodontal disease. However, Alfant *et al.* were interested in MMP levels in children with AgP and compared the results with healthy unrelated children and adults with ChP (Alfant *et al.*, 2008). Skurska *et al.* evaluated the effect of additional ozone therapy on MMP levels between adult patients with ChP and AgP (Skurska *et al.*, 2010). The definition of periodontal disease in the present study was based on clinical and radiological signs of periodontitis, and the requirement that at least four teeth with PPDs ≥ 6mm at the first visit had to be fulfilled by the patient to be included in the study. In the literature on biomarkers, a wide range of definitions of periodontitis and progression of periodontitis is used. According to the European Federation of Periodontology (EFP) the presence of proximal attachment loss of ≥3 mm in ≥2 non-adjacent teeth is already sufficient for a patient to be diagnosed as suffering from periodontitis (Tonetti and Claffey, 2005). To identify only cases with considerable extent of periodontal disease, stricter criteria for inclusion are adopted in most of the studies, including radiographic alveolar bone loss or a combination of clinical parameters. In the present study, the sex of the patient was no exclusion criterion and the group was evenly divided, with females constituting 55% of study population. By contrast, Kraft-Neumärker *et al.*, who performed analysis of full mouth profile of active MMP-8 in periodontitis patients, and Reinhardt *et al.*, who investigated the association of biomarkers in GCF with progression of periodontitis, included only females, in the latter case postmenopausal (Kraft-Neumarker *et al.*, 2012, Reinhardt *et al.*, 2010). Hormonal status and hormonal

fluctuations are associated with bone loss and thus the criterion might have influenced the biomarker levels and study results (Page *et al.*, 1997). In the substudy we did not stratify patients by their smoking status, where smokers constituted 55% of the study population. Tobacco smoking modifies the host response to bacterial challenge and periodontal treatment outcome is less favourable in smokers (Page and Kornman, 1997). Therefore Ozcaka *et al.* divided their study population by smoking habit to compare biomarkers levels, however, they did not find any differences in serum MMP-8 concentrations (Ozcaka *et al.*, 2011). Leppilahti *et al.* could cluster high baseline GCF MMP-8 levels in smokers, who showed poor response to periodontal treatment (Leppilahti *et al.*, 2014b). Similarly, Mäntylä *et al.* concluded in their prospective study that especially in smokers repeatedly elevated MMP-8 concentrations in GCF indicated sites at risk for disease progression (Mäntylä et al., 2006). In the present study, due to the blinding in clinical trial design, it was not possible to provide equal allocation to groups and to differentiate between patients who received antibiotics and those with placebo. In two intervention studies, researchers evaluated the association between antimicrobial therapy and the level of MMP-8. In RCT with subantimicrobial dose of doxycycline Reinhardt *et al.* found an association between increased amount of GCF MMP-8 during the first year of maintenance and increased odds of attachment loss after 2 years in the mixed population, but they observed no association when only SDD group was evaluated (Reinhardt *et al.*, 2010). The AgP group in the study of Skurska *et al.* had the highest initial level of mean salivary MMP-8 in comparison to ChP groups, but the changes after treatment by SRP with or without additional ozone therapy did not lead to statistically significant changes in the enzyme levels (Skurska *et al.*, 2010). In our study the primary goal was to evaluate the predictive ability of MMP-8. Such a test might be competitive with the current diagnostic tools only if it could be used in a wide range of patients and different treatment scenarios suggested by dental practitioners. Therefore, and also due to the requirements of the main study, we did not use more rigid criteria for the definition of periodontitis, nor did we select patients by the criterion of sex, smoking habit, adjunctive therapy or history of previous periodontal treatment. This inconsistency of study population might have contributed to the lack of statistically significant differences in our results obtained in the majority of the analysed parameters.

Table XXX: Differences in materials and methods in human studies evaluating matrix metalloproteinase-8 levels

Reference	Study sample	Smoking habit	Antimicrobial therapy	Source of MMP-8	Level of analysis	Form of detected MMP-8	Quantification method	Biochemical test	Localisation of testing unit	Panel of biomarkers
Lee *et al.*, 1995	17 G with no attachment loss 27 stable P 14progressive P	?	?	GCF	patient level/ 6 sites pp	separately active + latent MMP-8	collagenase units (µg/min)	SDS-PAGE fluorography	laboratory	-
Romanelli *et al.*, 1999	25 C 17 G 12 P	?	?	mouthrinse	patient level	separately latent, activated and superactivated forms	relative amount/ 50µL sample	SBA, Western blot	laboratory	MMP-8, MMP1, MMP-13
Mancini *et al.*, 1999	32 C (17 G,15 H) 125 AgP 5 EOP 1 Edentulous	?	with or without adjunctive AB therapy	mouthrinse, saliva	patient-level	separately active + latent	nano units/ sample	SDS-PAGE fluorography, SBA assay	laboratory	-
Kinane *et al.*, 2003	20 untreated chronic adult P	?	-	GCF	site-level/ 4 sites pp	Active	ng/ sample in 30s + ng/µL	IFMA	laboratory	-
Mäntylä *et al.*, 2003	8 H 10 G 11 P	?	-	GCF	site-level/ 58 g, 90p, 59c	Active	µg/L	MMP-8 test stick based on the immunochromato-graphy, IFMA	chair-side + laboratory	-
Miller *et al.*, 2006	29 H 28 gen.mod.-sev.P	33.3% gen.mod.-sev. P, 27.6% H	-	Unstimula-ted whole saliva	patient-level	Total	ng/mL	human Quantikine MMP-8 ELISA kit, R&D Systems, Minneapolis	laboratory	IL-1β, MMP-8, OPG
Mäntylä *et al.*, 2006	16 ChP	11	-	GCF	site-level/ 132 sites	not completely selective or specific for the active form of MMP-8	µg/L	MMP-8-specific periodontal chair-side dip-stick test, IFMA	chair-side + laboratory	-
Munjal *et al.*, 2007	?	?	?	GCF	site-level/ 15 h, 7 g, and 12 p sites	active, active, ?	ng/mL	DentoAnalyzer, IFMA, in-house ELISA	chair-side + laboratory	-
Prescher *et al.*, 2007	?	?	?	GCF	site-level/ 21h, 18 doubtful, 25 p	Active	ng/mL	DentoAnalyzer	chair-side	-

Golub *et al.*, 2008	128 P post-menopausal ♀	some	64 placebo, 64 SDD	GCF	patient level/ pooled from 2 sites	total	percentage of the total collagenase protein in GCF	Western blot	laboratory	MMP-1, MMP-8, MMP-13, ICTP, IL-1β
Rai *et al.*, 2008	15 H 18 G 20 P	?	?	saliva	patient-level	?	ng/mL	Human Quantikine MMP-8 ELISA kit	laboratory	salivary MMP-8, crevicular MMP-2, MMP-9
Alfant *et al.*, 2008	23 AgP +9 H siblings + 12 H unrelated African American children 12 ChP adults	some	-	GCF	site-level/ 2 sites pp	Active	ng/μL	commercially available fluorometric MMP kits specific for each MMP	laboratory	MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13
Xu *et al.*, 2008	4 H 5 G 10 P 5 I 5 PI	-	-	GCF, PISF	site-level/ 89 sites	?	per site + per μL	DNP-octapeptide, Western blot	laboratory	-
Ramseier *et al.*, 2009	18 H 32 G 28 mild ChP 21 sev. ChP	0% H, 19% G, 36% mild ChP, 81% sev. ChP	-	saliva	patient-level	Total	ng/mL	ELISA by R&D Systems, Minneapolis, MN	laboratory	MMP-8, MMP-9, calprotectin, OPG, IL- 1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, IFN-□ ,ICTP, *A. a., C. rectus, F. nucleatum, P. intermedia, P. gingivalis, T. forsythia, T. denticola*
Sorsa *et al.* 2010	2 H 2 G 6 P	some	-	GCF	Site level/ 20h, 18g, 45p	active, active, active, total	ng/mL	dentoAnalyzer, IFMA, the MMP-8 specific immunochromatographic chair-side dip-stick, Amersham ELISA	chair-side + laboratory	-

Gursoy *et al.*, 2010	81 C 84 P	17,2% C, 52,3% P	?	saliva	patient-level	active, total	ng/mL	IFMA, commercial ELISA kit by Amersham, GE Healthcare, Buckingamshire,UK	laboratory	MMP-8, MMP-14, TIMP-1, ICTP
Reinhardt *et al.*, 2010	128 mod-sev.P post-menopausal osteopenic ♀	20%	64 SDD, 64 Placebo	GCF	patient-level/ 2 sites pp	active + total	units/ sample	total collagenase activity using hydrolysis of a synthetic octapeptide Western blot	laboratory	IL-1β , MMP-8, ICTP
Hernandez *et al.*, 2010	25 mod.-sev. ChP	?	-	GCF	site-level/ 25 active sites, 25 inactive sites	separately PMN MMP-8 pro, active, % activation, mesenchymal MMP-8 pro, active, % activation, MMP-8 complexes, fragments, total	ng/mL	Western blot, IFMA	laboratory	MMP-8, MMP-14, MPO, TIMP-1
Skurska *et al.*, 2010	12 CP-S 25 CP-O 15 AP 14 C	?	-, SRP, SRP + ozone therapy	saliva	patient-level	Total	ng/mL	Human Quantikine MMP ELISA kit by R&D Systems, Minneapolis, MN, USA	laboratory	MMP-1, MMP-8, MMP-9
Marcaccini *et al.*, 2010	27 ChP 15 C	-	-	GCF	site level= patient level	Total	ng/site in 30s	ELISA kit by DuoSet R&D Systems Inc., Minneapolis, MN, USA	laboratory	MMP-8, TIMP-1, TIMP-2, MPO, MMP-9
Kinney *et al.* , 2011	18 H 32 G 28 mild ChP 21 mod.-sev.ChP	0% H, 19% G, 36% mild ChP, 81% sev.ChP	-	saliva, serum	patient-level	Total	log^2 pg/mL	ELISA by R&D Systems, Minneapolis, MN	laboratory	salivary OPG, MMP-9, MMP-8, IL-1β, calprotectin, ICTP, serum OPG, MMP-9, MMP-8, IL-6, calprotectin, ICTP, CRP, TNF-α, biofilm pathogens
Ozcaka *et al.*, 2011	56 H 55 ChP	17 C, 16 ChP	-	serum	patient-level	Active	ng/mL	IFMA	laboratory	MMP-8, MPO, MMP-9, TIMP-1, NE
Leppilahti *et al.*, 2011	36 H 21 mild IB 104 mod IB 53 strong IB	some	-	oral rinse	patient-level	active, active, total	ng/mL	dentoELISA, IFMA, commercial ELISA by Amersham	laboratory	MMP-8, TIMP-1, elastase activity

Sexton *et al.*, 2011	68 ChP	23% of the SRP group, 33% of the OHI group	-	saliva	patient-level	total	ng/mL	human quantikine ELISA kits by R&D Systems, Minneapolis, MN, USA	laboratory	IL-1β, IL-8, MIP-1□, TNF, OPG, MMP-8
Konopka *et al.*, 2012	21 H 30 gen. advanced ChP	-	-	GCF	patient level	Total	ng/sample	commercial ELISA by Quantikine R&D Sys- tems Inc., Minneapolis, MN, USA	laboratory	IL-1β, IL-8, MMP-8
Kraft-Neumärker *et al.*, 2012	9 ChP ♀	-	-	GCF	patient-level + site-level/ 92-112 sites pp	Active	ng/mL	ELISA using specific monoclonal antibodies.- 8708 and 8706	laboratory	-
Emingil *et al.*, 2012	32 gen. AgP	43.8% of azithromycin group, 38.5% of placebo	16 azithromycin 16 placebo	GCF	patient level= site-level	Active	pg/sample + concentration	IFMA	laboratory	MMP-8 ,TIMP-1 *A.a., P. gingivalis, T. forsythia, F. nucleatum, P. intermedia* and total bacteria
Rathnayake *et al.*, 2013	451 random patients	13.5- 28.6%	?	stimulated saliva	patient-level	Active	ng/mL	IFMA	laboratory	IL- 1β, IL-6, IL-8, lysozyme, MMP-8, TIMP-1
Gursoy *et al.*, 2013	81C 65 loc. P 84 gen. P	19C, 19 loc. P, 48 gen. P	?	saliva	patient-level	Active	ng/mL	IFMA	laboratory	MMP-8, MMP-9, MMP-13, TRACP 5b, CTx, NTx, ICTP
Goncalves *et al.*, 2013	29 loc. AgP	-	amoxicillin + metronidazole	GCF	site-level/ 1 diseased site +1 healthy site pp	Active	ng/mL	fluorometric MMP kits specific for each MMP	laboratory	MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13
Salminen *et al.*, 2014	340 G-mild P, 123 mod.-sev.P 30 Edentulous	7.9% G-mildP, 22.8% mod.-sev.P, 10.3% Edentulous	some	saliva	patient-level	Active	ng/mL	IFMA	laboratory	MMP-8, IL-1β, *P.gingivalis*
Leppilahti *et al.*, 2014a	9 H 6 G 8 P	4 H, 3 G, 5 P	-	GCF	site-level/ 20h,19g, 19p	active, total	ng/mL	IFMA, commercial Biotrak ELISA system by GE Healthcare, Amersham	laboratory	azurocidin, chemokine ligand 5, MPO, TIMP-1 MMP-13, MMP-14

Leppilahti *et al.,* 2014b	15 ChP	10	-	GCF	site-level, patient-level/ 5-7 sites pp	active	ng/mL	IFMA	laboratory	-
Kinney *et al.*, 2014	18 H 32 G 28 mild ChP 21 sev. ChP	0% H, 19% G, 36% mild ChP, 81% sev.ChP	-	GCF, saliva, serum	patient-level/ 8 highest-ranked sites from mesiobuccal aspect of each toothsite	Total	pg/mL	ELISA by Quantibody Human Cytokine Array by RayBio-tech, Inc., Norcross, GA, USA	laboratory	MMP-8, MMP-9, OPG, C-reactive protein, IL-1β, biofilm pathogens
Wohlfahrt *et al.*, 2014	16 PI OFD+EDTA, 16 PI OFD+EDTA+ titan granules	?	Amoxicillin and metronidazole	PISF	patient-level/ 4 sites per implant	Total	pg/ mL	Quantikine Human Total MMP-8 (DMP800) ELISA	laboratory	MMP-8, IL-6, OPG, osteocalcin, leptin, osteopontin, parathyroid hormone,TNF-α, adiponectin, insulin
Pourabbas *et al.*, 2014	22 mod.-sev. ChP	-	SRP or SRP + PDT	GCF	site-level/ 1 SRP site + 1 SRP + PDT site pp	Total	pg/µL	sandwich ELISA	laboratory	IL-1β, TNF-α, MMP-8, MMP-9
Skurska *et al.*, 2015	18 AgP with SRP+AB 18 AgP with SRP +AB	?	amoxicillin + metronidazole, PDT	GCF	site-level= patient level/ 1 deepest PD pp	Total	concentration	commercially available kits by R&D Systems, Minneapolis, MN, USA	laboratory	MMP-8, MMP-9
Ramseier *et al.*, 2015	? patients with implants	some	?	PISF, GCF	site-level/ 1 deepest site at 504i + 493 adjacent teeth	Total	pg/site	commercially available ELISA kits by R&D Systems Europe Ltd, Abingdon, UK	laboratory	IL-1β, MMP-3, MMP-8, MMP-1, MMP-1, TIMP-1
Rathnayake *et al.*, 2015	200 patients with a first MI 200 C	10% MI (5% snuffing), 3% non-MI (12% snuffing)	2% MI and 5% non-MI on anti-inflammatory drugs	saliva	patient-level	pro MMP-8, active MMP-8	ng/mL, arbitrary unit	IFMA	laboratory	MMP-8, MMP-9, MPO, TIMP-1

Leppilahti *et al*., 2015	67 ChP 32 gen. AgP	13 gen. AgP sites, 73 ChP sites	116 site none, 30 sites azithromycin 12 sites LDD	GCF	site- level/ 1 or more sites pp ,158 sites	Active	normalized MMP-8 levels: % values from the population maximum	IFMA	laboratory	-
Izadi Borujeni *et al*., 2015	15 gen.mod.ChP 15 gen.sev.ChP 30 C	30%ChP, 53% C	-	oral rinse	patient-level	Active	ng/mL, dichotomous: + (≥25 ng/mL) or -	lateral flow-sandwich-test	chair-side	-

MMP = matrix metalloproteinase; G = gingivitis; P = periodontitis; C = controls; H = healthy; AgP = aggressive periodontitis; EOP = early-onset periodontitis; gen. = generalised; loc. = localised; mod. = moderate; sev. = severe; ChP = chronic periodontitis; ♀ = females; I = implants; PI = peri-implantititis; SRP = scaling and root planing; CP-S = patients with ChP, who underwent SRP; CP-O = patients with ChP who additionally to SRP underwent ozone therapy; AP patients with AgP who additionally to SRP underwent ozone therapy; IB = inflammatory burden; OFD = open flap debridement; EDTA = ethylenediaminetetraacetic acid; AB = antibiotics; MI = myocardial infarction; OHI = oral hygiene instructions; SDD = subantimicrobial dose doxycycline; PDT = photodynamic therapy; LDD = low-dose doxycycline; GCF = gingival crevicular fluid; PISF = peri-implant sulcus fluid; pp = per patient; PD = pocket depth; SDS-PAGE = sodium dodecyl sulfate olyacrylamide gel electrophoresis; SBA = soluble biotinylated collagen assay; IFMA = immunofluorometric assay; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; OPG = osteoprotegerin; ICTP = pyridinoline cross-linked carboxyterminal telopeptide of type I collagen; TNF = tumor necrosis factor; IFN = interferon; TIMP = tissue inhibitor of matrix metalloproteinase; MPO = myeloperoxidase; CRP = C-reactive protein; NE = neutrophil elastase; TRACP 5b = tartrate-resistant acid phosphatase serum type 5b; CTx = C-terminal cross- linked telopeptide of type I collagen; NTx = N-terminal cross-linked telopeptide of type I collagen

5.2 Plaque Index

It is not possible to achieve a total absence of dental plaque, neither supra- nor subgingivally. The goal of periodontal therapy with regard to plaque is to reduce the total number of periodontal pathogens, to change the biofilm composition and to restore the homeostatic balance between the bacterial community and the host. Oral hygiene measures alone are not sufficient in the treatment of advanced periodontitis, but without proper oral hygiene level, more advanced periodontal therapies are less effective and further attachment loss may follow (Corbet and Davies, 1993). Several methods of assessment of supragingival plaque level and oral hygiene level are used by different researchers. The most commonly used indices are the simplified Plaque Index by O'Leary (1972) and Approximal space Plaque Index by Lange (1977); both dichotomous and expressed in 0-100%, or Plaque Index by Silness and Löe (1964) calculated as mean from values in a scale 0-3. In our studies we used the API by Lange during the oral hygiene phase at pre-treatment appointments. Initial plaque levels before any oral hygiene counselling can reach up to 100% in periodontally affected patients. We aimed at reaching the value of API ≤ 25%, which is considered an optimal oral hygiene level, and it took us up to 4 visits to achieve this in our patient group. Starting from the baseline measurement we used the PI by O'Leary because of the study protocol and settings of electronic periodontal probe. The mean value of PI by O'Leary in our study group was 11%, which was very low in comparison with other studies, where plaque level at baseline measurement was typically above 80%, as in the work of Hernandez *et al.* (PI = 100%), Skurska *et al.* (API = 92.90%, PI = 1.89), Emingil *et al.* (plaque = 98.73%) which is considered poor or insufficient (Skurska *et al*., 2010, Hernandez *et al*., 2010, Emingil *et al*., 2012). The discrepancies come from the fact that first oral hygiene instruction in the cited studies was provided only during SRP visit, so already after baseline measurement. As a consequence, due to already very low PI established at baseline visit, excellent patients' compliance and success in patients' motivation and remotivation during periodontal therapy, the PI in our study group remained stable and did not change statistically significantly during subsequent visits. By contrast, other researchers showed a significant reduction in plaque level, reaching in the course of therapy moderate to optimal values, which are more consistent with our results; plaque= 25.07%, PI = 24%, API = 31.04% and PI= 0.47 (Emingil *et al*., 2012, Hernandez *et al*., 2010, Skurska *et al*., 2010).

5.3 Periodontal pocket depth and clinical attachment level

The precision of measurements of clinical parameters depends on one hand on the accuracy of the scale of a periodontal probe, on the other hand on the precision of measurement itself and on applied forces. There is a variety of periodontal probes accepted for periodontal measurements and thus used in different studies evaluating the level of MMP-8 in periodontally affected patients. Classical manual instruments, scaled with an accuracy of 1 mm were used in some studies (Skurska et al., 2010, Mäntylä et al., 2006, Hernandez et al., 2010, Konopka et al., 2012, Emingil et al., 2012). In our study, we used automated, force controlling probe with an accuracy of 0.2 mm and calibrated for 0.25 N pressure to maximise the precision of the measurements of periodontal pocket depth and to minimise the risk of inadequate measuring force. Similarly, Florida Probe was chosen by some authors (Kinane *et al*., 2003, Marcaccini *et al*., 2010). Like other researchers, we recorded a classical, full mouth profile of pocket depth, based on 6 measurement points per tooth. As we were interested in predicting periodontal disease at the patient level, we calculated a mean pocket depth value based on all measurement points per patient and compared the medians from all patients between subsequent time-points. We used the median instead of mean for comparison whenever possible, as it is more resistant to outliers. As a result, we obtained a median pocket depth of 3.27 mm at the baseline. These results are in line with the results of Hernandez *et al.* who reported a mean of 3.40 mm ± 1.21 mm in the group of chronic periodontitis patients (Hernandez *et al.*, 2010). Emingil *et al.* showed higher baseline values; 95% confidence interval values of 4.05 - 4.81 mm and 3.79 - 4.31 mm depending on the group – it might be explained by the fact that she included only patients with generalised aggressive periodontitis in her study, whereas we had a heterogenic group of chronic and aggressive periodontitis patients, in both local and generalised form (Emingil *et al.*, 2012). Other studies show much higher initial probing depth values of up to 6.4 ± 0.6 mm (Konopka *et al.*, 2012). It has to be stressed, however, that these authors, even if they used full-mouth scoring of periodontal parameters, they performed the statistical analysis only of the sites which were subjected to MMP-8 sampling. As a result, their mean PD value per patient was calculated based only on 1 tooth by some authors, as in Marcaccini *et al.*, up to inexact number of tooth sites in other studies, where only pathologically affected pockets, mostly PD ≥ 5mm, were included in the analysis (Marcaccini et al., 2010, Skurska et al., 2010, Mäntylä et al., 2006). Our data were thus more precise in representing the full

mouth profile of PD in each patient, at the same time, however, the obtained median value of 3.27 mm is still considered a value representing periodontal health. It is a result of the site-specific nature of periodontal disease with typical coexistence of multiple sites representing physiological sulcus depth, that is, values up to 3.5 mm, and pathologically deepened pockets. Full mouth profile is based on up to 192 points in case of a fully dentured patient and 6 measurement points per tooth. As a result, statistical analysis of full mouth profile make a few extreme values disappear among the majority of shallow depths, even in the case of generalised form of periodontitis, and that was the case in our patient group. The longitudinal changes in mean PD in our study correspond with the results of other authors and classical studies which show the most drastic reduction in PD at the next visit following SRP, scheduled between 1 week and 3 months after the SRP visit (Kinane *et al*., 2003, Emingil *et al*., 2012, Marcaccini *et al*., 2010). After that, the values tend to stay on the same level or to slightly decrease, if maintenance therapy is implemented, both in the pure SRP patients as well as in patients with additional antimicrobial therapy. The initial, highest PD value at visit 2, as representation of the highest inflammation level, was never reached again during further measurements during our study. Like in other studies, we also saw the greatest improvement between baseline visit 2 and following measurement visit after 4 months, and good improvement between 7^{th} and 10^{th} months. The lack of statistical changes in PD between 4^{th} and 7^{th} month was expected and can be explained by the lack of mechanical periodontal treatment, not performed at previous visit, visit number 4, because of the main study protocol. The same characteristics of longitudinal changes and stabilisation between 4^{th} and 7^{th} month can be observed for the CAL, which is influenced mostly by changes of PD, rather than by recession, partially due to the fact, that most of our patients underwent rigorous pre-treatment phase, when signs of marginal gingival inflammation were eliminated before the baseline visit.

5.4 Bleeding on probing

Bleeding on probing defined in most of the studies is calculated from both bleeding from physiological sulcus, which represents gingivitis, and bleeding from a periodontally deepened pocket. As a result, the final value represents the mixed inflammation level of both superficial tissues and deeper layers. Proper oral hygiene regime and prophylactic sessions are the key factors to reduce bleeding from the gingival sulcus (Loe *et al.*, 1965). In our study, we emphasised the role of the oral hygiene phase, whose goal was

to reduce the PBI, before proceeding to the baseline periodontal measurement and mechanical treatment. As a result, the BOP values in our baseline measurement resulted mainly from the bleeding originating from periodontal pockets, and sulcular bleeding was non-essential. Consequently, the baseline BOP values in our study population equal to 26% were extremely low in comparison with other studies, where oral hygiene instruction and prophylactic treatments were introduced only after the baseline measurements. In some studies BOP before periodontal pre-treatment was found in up to 100% of measured sites (Hernandez *et al.*, 2010). In our study, as well as in other analysed studies, the BOP values were reduced significantly during the course of periodontal treatment, with the strongest drop occurring directly after the initial therapy (Skurska *et al*., 2010, Emingil *et al*., 2012, Kinney *et al*., 2011). Between 4th and 7th month after the baseline, there was a slight increase of the parameter in our study from 14% to 18% indicating clinically more inflammation. This could be expected, as no mechanical treatment was performed during visit 4 because of the main study protocol. At consecutive visit 6, the BOP already stays stable, which can be explained by the performed treatment at previous visit. In the listed studies, there is a trend to continuous reduction of the mean BOP over time; this can be explained by typical study protocols, where during each measurement visit a mechanical treatment is performed, contrary to the particular protocol of our study. The final value of 17% after 10 months of treatment is in line with data of other authors, as with 14.17- 21.61% in azithromycin group or 15.60 - 22.33% in placebo group achieved by Emingil *et al.* after 6 months of treatment or 16% to 28% depending on the site activity by Hernandez *et al.* after periodontal treatment (Hernandez *et al*., 2010, Emingil *et al*., 2012).

5.5 Levels of MMP-8

5.5.1 Source of MMP-8

Bacteria are a necessary, but not a sufficient factor for the development of periodontitis (Kinane, 2001). Their enzymes are only to a small degree responsible directly for the cleavage of periodontal collagen and soft tissue breakdown. Clinical parameters such as pathologically deepened sulci which became periodontal pockets, the loss of clinical attachment due to pockets or gingival recessions, and finally BOP do not destroy the periodontal collagen fibres. They can be predictors of periodontal stability or indicate the risk of disease progression, but it is the proteolytic enzymes, such as MMPs, which form the inflammatory cascade and directly cleave the collagen, leading to the degradation of

soft and hard tissue matrix (Lang *et al*., 1986, Lang *et al*., 1990, Sorsa *et al*., 2010, Leppilahti *et al*., 2011, Gursoy *et al*., 2010). MMP-8, the key host cell collagenase during periodontal inflammation with its unique ability to destroy collagen types I and III, can be detected in various body compartments, with GCF and saliva being the most frequently studied, as seen in Table XXX (Sorsa *et al*., 2006, Yucel-Lindberg and Bage, 2013). We chose the GCF as a source of the biomarker, as the volume and composition of GCF reflect most accurately the current physiological or pathological processes taking place in the given periodontium, as it is actually the filtrate from circulation, permeated through diseased soft tissue (Kinane *et al*., 2003). As periodontal disease is a site-alternating and intermittent disease of bursts of exacerbations and stable periods we should ideally sample every tooth site to reflect the overall patient's actual condition most accurately. This effort has been made by Kraft-Neumarker *et al.,* who sampled four sites from every single tooth in the group of nine female patients with chronic generalized periodontitis, resulting in 92-112 sites per patient. Sampling by an experienced dentist took 10-15 min. per patient, which in private dental office settings is not really feasible due to the required time and financial effort (Kraft-Neumarker *et al.*, 2012). In our studies we were looking for the efficient method, which could be realistically performed on the wide scale in dental surgery: that is why we chose four sites per patient. Other authors sampling MMP-8 from GCF used miscellaneous protocols, from only one site per patient, such as Marcaccini *et al.*, Emingil *et al.* or Skurska *et al.*, through 2-6 sites per patient, which was the most common, up to 28 sampling sites per patient, as did Kinney *et al.*, 2014 (see Table XXX) (Marcaccini *et al*., 2010, Emingil *et al*., 2012, Skurska *et al*., 2015, Kinney *et al*., 2014). Sampling only at few random sites and extrapolating the results on the whole patient carries the risk that we by chance sample e.g. only the pockets, which are currently not in a state of active inflammatory process, and there might be pockets at other tooth sites that are responsible for the overall classification of a patient's periodontal status. This problem occurs in every screening system and a study designed to answer the question as to the minimum sampling sites per patient needed to reflect adequately his or her general periodontal status would be useful. Since in our study the sampling sites were predetermined at the baseline visit, the situation arose, that in some cases we sampled sites which normally would not be classified as periodontally affected. This can be explained by the fact that the oral hygiene phase aiming to reduce gingivitis was performed after the allocation of the sites, and by the site-fluctuating nature of

periodontal disease. We classified already the sites of 4mm PD as periodontally involved, as they already require a treatment according to the protocol that is currently accepted worldwide and are thus subject to MMP-8 sampling. However, now we reckon that it would be safer to choose only the sites with manifestly severe periodontal involvement, that is clinically with PD≥ 6mm and radiological bone loss, as done by Skurska *et al.,* who was looking for the deepest site in every patient, Emingil *et al.*, who chose the site with PD≥ 6mm or Mäntylä *et al.*, who sampled sites with PD≥ 4mm, but only with simultaneous radiographic bone loss (Skurska et al., 2015, Emingil et al., 2012, Mäntylä et al., 2003). Numerous authors were able to show differentiation in MMP-8 levels between healthy patient and patients with severe periodontitis, but often this difference between healthy or gingivitis group and patients with mild or moderate periodontitis faded (Ramseier *et al*., 2009, Leppilahti *et al*., 2014a, Salminen *et al*., 2014). By using stricter inclusion criteria of sampling sites we could get a wider separation between clearly healthy and clearly diseased sites and thus the median MMP-8 values obtained from sites classified as diseased might have been higher. The depth of the insertion of a sampling device, sampling time and sample processing might influence the final result. As described in Materials and Methods, we used the paper strips, inserted for 30 seconds only to the first 2 mm of the crevice and sent the vials directly to the laboratory, as recommended in the manual of the analysing centre. Kinane *et al*., Kraft-Neumärker *et al.*, Leppilahti *et al.*, Skurska *et al.* had similar sampling protocol, but other authors, like Goncalves *et al.* or Golub *et al.* shortened the sampling time up to 10 seconds (Kinane *et al*., 2003, Kraft-Neumarker *et al*., 2012, Leppilahti *et al*., 2015, Golub *et al*., 2008, Goncalves *et al*., 2013). Other authors inserted the stripes until light resistance was felt which suggests the full depth of the crevice (Hernandez *et al*., 2010, Konopka *et al*., 2012, Emingil *et al*., 2012). This may result in obtaining different quality and quantity of the GCF samples and thus a different result. The result depends also on different quantification methods used; the most common was data representation as concentration, e.g. nanograms per millilitre, as in our study. Picograms or micrograms can be easily converted mathematically and compared, but some authors used instead arbitrary units, absolute values per site or percentage values from the population maximum, which makes direct comparison difficult, see Table XXX (Konopka *et al*., 2012, Romanelli *et al*., 1999, Ramseier *et al*., 2015, Leppilahti *et al*., 2015). Interestingly, certain researcher groups measured in their studies the MMP-8 levels expressed both as total amount in a sample and as a

concentration, and this influenced the results. Emingil *et al.* showed that 3 months after treatment the levels were reduced statistically significantly when expressed as total amount, but not as a concentration (Kinane *et al*., 2003, Emingil *et al*., 2012). The amount of GCF can be influenced by the local level of inflammation, by the smoking habits and it exhibits a patient-to-patient variation which might possibly explain the differences. GCF sampling offers us the choice of the level of analysis; one site can be treated as a separate unit so a site-level analysis can be performed, or all the samples or the data from all samples from one patient can be pooled, the mean calculated and a patient-level result obtained. Again, different methods were chosen by different authors, as presented in Table XXX. In cross-sectional part of our study we chose the site-level analysis for direct comparison between clinical parameters and MMP-8 levels, and to check the ability of MMP-8 to predict the disease activity we included the patient-level analysis. As it is not feasible in a dental office to perform a site-level analysis of multiple sites, we wanted to concentrate on the clinical applicability of the testing. Saliva, or its diluted form, a mouth rinse, was the second most frequently chosen sampling source for MMP-8 by many researchers; Gursoy *et al.*, Rathnayake *et al.* or Salminen *et al.* investigated the association of salivary biomarkers with periodontal parameters and Sexton *et al.* assessed their changing levels longitudinally to determine the response to a therapy (Gursoy *et al*., 2013, Rathnayake *et al*., 2013, Salminen *et al*., 2014, Sexton *et al*., 2011). Saliva *per se* represents a pooled sample and the result on a patient-level, it is easy, non-invasive and takes little time to collect. However, saliva represents a complex fluid mixture, and gingival crevice exudate accounts only for a part of its composition. The flow rate fluctuates during the day and depends strongly on the stimuli affecting salivary glands, hormonal levels or intake of medications which dictates the concentration of its constituents such as MMP-s. Besides that it remains unclear if salivary biomarkers are able to distinguish the disease if it has only localised character or is of a mild grade, which happens often in periodontally affected patients. Kinney *et al.* showed in his study population of periodontally healthy patients, of patients with gingivitis, mild periodontitis and moderate/severe periodontitis that salivary test had better sensitivity, whereas GCF biomarkers showed better specificity in the identification of periodontally progressing patients (Kinney *et al*., 2014, Kinney *et al*., 2011). In the case of patients with dental implants, the amount of implants inserted usually does not exceed ten in fully implant-supported cases, in comparison with 32 teeth in fully dentated person. These are often patients with a history of periodontitis and a higher

risk of development of peri-implant disease. Therefore, it might be useful and clinically practicable to perform a site-level analysis of the putative biomarkers of peri-implant disease, such as MMP-8. Ramseier *et al.* did not report any differences in median levels of MMP-8 between implants and adjacent teeth, whereas Xu *et al.* detected increased concentration of collagenase in PISF from peri-implantitis sites by 971% in comparison to healthy implant sites (Ramseier *et al*., 2015, Xu *et al*., 2008).

5.5.2 Levels of MMP-8 in different quantification methods

Previous authors reported different levels of MMP-8 depending on patient's periodontal status. Romanelli *et al.* in their study confirmed that MMP-8 is a primary collagenase found in patients with chronic periodontitis who had 6-fold higher levels of collagenase activity compared to patients with gingivitis. They used soluble biotinylated collagen assay (SBA) and Western blotting to assess the collagenolytic activity and molecular forms of MMP-8 and the data was expressed in nanounits (Romanelli *et al.*, 1999). Mancini *et al.* used a similar SBA assay and showed that MMP-8 activity was 18 times higher in severe periodontitis patients than in stable maintenance patients or in healthy controls. They adopted the threshold of 80 nanounits to distinguish severe periodontitis cases from moderate ones (Mancini *et al*., 1999). Mäntylä *et al.* applied the laboratory time-resolved immunofluorometric assay (IFMA) and compared it with the MMP-8 dip-stick test, which is based on immunochromatography and which resembles a pregnancy test (Mäntylä et al., 2003). Both tests use two monoclonal antibodies which detect neutrophil and non-neutrophil-types of MMP-8 isoforms, especially their active form. They calculated a cut-off level of GCF MMP-8 of 1000 µg/L (1 µg/mL) to distinguish the periodontitis sites from gingivitis or healthy ones. Prescher *et al.* tested a novel device, the DentoAnalyzer, which performed a rapid quantitative chair-side analysis for active MMP-8 in GCF (Munjal et al., 2007). They reported a median level of 1ng/mL for healthy sites, 6.3 ng/mL for doubtful sites, and 14.3 ng/mL for periodontitis sites (Prescher *et al.*, 2007). However, both groups – Mäntylä *et al.* and Prescher *et al.* – used different elution and dilution protocols for their samples and thus the data cannot be directly compared. By dividing the numbers obtained by Mäntylä *et al.* from IFMA by 70, the results obtained of 14 ng of aMMP-8/mL of eluate for the cut-off value can be directly comparable with the data of Prescher *et al.* using the DentoAnalyzer (Sorsa *et al.*, 2010). Remarkable is the broad distribution of the data from the samples. Prescher observed a range of 0.0–7.4 ng/mL for healthy sites, 0.0–27.1 ng/mL for doubtful sites

and 5.7–64.6 ng/mL for sites classified as periodontally affected. This is in line with our data, where the median aMMP-8 for periodontitis sites was 5.00 ng/mL, but we obtained the broad range of values from 0.59 ng/mL up to 81.87 ng/mL at the initial visit, with a high standard deviation which exceeded the mean value. This problem was also observed by Kraft-Neumarker *et al.* who reported the mean concentration of aMMP-8 in their periodontitis female patients from a low level of 3.2 ng/mL of eluate up to 23.7 ng/mL of eluate (Kraft-Neumarker *et al.*, 2012). In their study both the pocket depth and the levels of aMMP-8 in GCF differed substantially not only between patients, but also between one site and another within one patient. Thus, five of nine periodontitis patients in that study had mean aMMP-8 values of < 8 ng/mL of eluate, so, based on aMMP-8 levels, they should be classified as periodontally healthy according to Prescher. The data of Kraft-Neumarker *et al.* are most adequate for comparison with our results, as they also used GCF as a sampling material, similar sampling depth of 2 mm and, what seems most important, they chose ELISA with specific monoclonal antibodies 8708 and 8706 for their laboratory analysis. These strong variations between patients and sites observed in our study as well as by other authors might be explained by the complex nature of periodontal disease, it's episodic character with stable and active periods, and we cannot determine correctly at which stage exactly the sampled site is. Furthermore, periodontitis is a multi-factorial disease with a wide variability of individual response to the microbial challenge. The protective role of MMP-8 and the persistence of physiologic levels of MMP-8 involved in down-regulation of inflammatory processes should also not be neglected. It seems difficult to set a rigid, common cut-off value for the whole population to differentiate between healthy and diseased sites, and it seems even more difficult to extrapolate it to the patient level. The differences of aMMP-8 levels between individuals can easily be as high as up to 7-fold values, despite similar clinical diagnosis as shown in the group of chronic generalized periodontitis female patients (Kraft-Neumarker *et al.*, 2012). Direct comparison between our data and those of other authors seems to be complicated due to already mentioned difference in biochemical tests used for detection and quantification of MMP-8. Classic Western blot and fluorographs used in older studies provided a more visual result or relative units for collagenase activity rather than numerical values (Lee *et al*., 1995, Romanelli *et al*., 1999). The great majority of authors did not provide information about the form of MMP-8 their laboratory methods quantified. The commercial ELISA kits from various companies used in many studies as presented in Table XXX, do not differentiate

between the active and latent forms of MMP-8 (Konopka *et al*., 2012, Miller *et al*., 2006, Rai *et al*., 2008, Ramseier *et al*., 2009, Skurska *et al*., 2010). Thus for example the values of median 203.8 ng MMP-8 per 1 mL of saliva of moderate to severe periodontitis patients measured with ELISA from R&D Systems reported by Ramseier *et al.* are difficult to compare with median 770.8 ng of active MMP-8 per 1 mL of saliva of generalised periodontitis patients measured with IFMA as presented by Gursoy *et al.* (Ramseier *et al*., 2009, Gursoy *et al*., 2013). Even within standard ELISA kits, when the sample was taken from GCF instead of saliva and the result was presented in nanograms per sample, as by Konopka *et al.,* the value of 18.6 ± 6.4 ng of MMP-8 per sample cannot be compared directly with the results of authors using different sources of enzyme and different scales (Konopka *et al.*, 2012). Various authors performed a comparison of different laboratory and chair-side methods to detect the levels of MMP-8 (Sorsa *et al*., 2010, Gursoy *et al*., 2010, Leppilahti *et al*., 2011). The overall conclusion can be drawn that IFMA, DentoAnalyzer or dentoELISA, which all use special monoclonal antibodies against active form of MMP-8, have after adjustment a high Spearman's correlation coefficient of e.g. 0.95 as shown by Sorsa *et al.* (Hanemaaijer *et al*., 1997, Sorsa *et al*., 2010). By contrast, the results obtained by commercial ELISA kits, on the contrary, were not in line with the findings of other methods.

5.5.3 Correlation of MMP-8 levels with clinical parameters

Clinical measurement of probing depths and signs of inflammation such as bleeding on probing are a recognized method to characterize the range of previous tissue breakdown in periodontitis patients. But these features are poor predictors of future tissue destruction and disease activity (Armitage, 2013). In our data we showed a weak correlation between initial PD and initial aMMP-8 measurement (r=0.18), however this interdependence was not statistically significant at other time-points. Ambiguous data has been published by other authors; Rai *et al.* reported correlation between salivary MMP-8 levels and PD (correlation= 0.42), Ramseier *et al.* obtained moderate correlation between MMP-8 and PD in GCF (r= 0.229), and between MMP-8 in PISF of adjacent implants (r= 0.243) (Rai *et al*., 2008, Ramseier *et al*., 2015). In the study of Marcaccini *et al.,* GCF MMP-8 levels correlated moderately with PD in chronic periodontitis patients (r=0.39) but not in control subjects (Marcaccini *et al.*, 2010). Konopka *et al.* showed contradictory results; in healthy subjects the MMP-8 levels correlated with PD (r= 0.53), but not in the group of chronic periodontitis patients (Konopka *et al.*, 2012). Others

reported correlation between MMP-8 levels and the number of pockets deeper or equal to 4 or 5 mm (Gursoy *et al*., 2013, Sexton *et al*., 2011). Rathnayake could correlate salivary active MMP-8 concentration with the number of pockets deeper or equal to 6 mm, but not with the total number of pockets (Rathnayake *et al.*, 2015). Alfant *et al.* found no significant correlation between GCF active MMP-8 concentration and PD, neither could Goncalves *et al.* at the site-level nor with mean PD in their group of local aggressive periodontitis children (Alfant *et al*., 2008, Goncalves *et al*., 2013). Deepened pocket does not necessarily mean a pocket with an active ongoing inflammation with histologically detectable transformation. As reported by Kraft-Neumarker *et al.*, deep pockets with low levels of aMMP-8 were present, likewise shallow pockets with high aMMP-8 levels (Kraft-Neumarker *et al.*, 2012).

In our study, due to lack of statistical significance, in none of the time-points could we correlate the presence of BOP with PD. This could be related to the fact that 55% of our patients were smokers, and the use of tobacco masks the clinical signs of inflammation expressed by BOP. Whenever BOP was present in our site-level analysis, the aMMP-8 levels were statistically significantly higher at those sites in comparison with sites without BOP, except for visit 5, where the difference did not reach a statistically significant level. The published data revealed mostly weak to moderate correlation between BOP and salivary levels of MMP-8 in periodontitis patients, stronger correlation of 0.58 was published by Miller *et al.* (Rai *et al*., 2008, Sexton *et al*., 2011, Miller *et al*., 2006, Rathnayake *et al*., 2013, Rathnayake *et al*., 2015). All correlation values between the levels of total MMP-8 in GCF or PISF and BOP were established below the threshold of 0.5 (Marcaccini *et al*., 2010, Ramseier *et al*., 2015). On the other hand, from the pockets equal to or deeper than 5mm, where the concentration of active MMP-8 was higher than 1 mg/l, 89% of the pockets also showed positive BOP.

5.5.4 Longitudinal changes in MMP-8 levels

Since the 1990s there is strong evidence, that active collagenase plays a direct role in the pathological destruction of periodontal tissue. In progressive lesions the levels of active form of collagenase can increase 33 times more than in stable lesions ($1{,}28 \times 10^{-4}$ collagenase units per day in progressive sites versus $3{,}87 \times 10^{-6}$ collagenase units per day in stable sites) (Lee *et al.*, 1995). Most authors observed longitudinal changes in MMP-8 levels in combination with performed periodontal treatment. In our study, where non-surgical periodontal treatment was performed, with or without additional oral

antibiotic therapy, the strongest decrease was observed within the first 2 weeks after non-surgical treatment, and afterwards during the maintenance visits the levels increased, however without reaching the initial values. Similar decrease by about a half within a few weeks - depending on the study outline - after SRP was observed also by other authors (Mancini et al., 1999, Mäntylä et al., 2003, Konopka et al., 2012). Results also depended on the system used for the detection of the enzyme; Sorsa reported a decrease of active MMP-8 value after SRP by 63.1% when measured with IFMA or by 71.4% with dentoAnalyzer, whereas when the same samples were analysed with Amersham ELISA, the reduction was not statistically significant (Sorsa *et al.*, 2010). Less pronounced reduction of salivary MMP-8 was observed by Kinney *et al.,* where the difference was statistically significant only for the moderate to severe periodontitis group, but not for mild periodontitis (Kinney *et al.*, 2011). Pourabbas *et al.* reported a decrease of GCF MMP-8 concentration by 11.07% (± 13.89%) in the SRP group, while patients with additional photodynamic therapy (PDT) reached a reduction by 21.22% (± 20.17%) (Pourabbas *et al.*, 2014). These results could not be confirmed by Skurska *et al.* who observed no statistically significant reduction of GCF MMP-8 levels in the patient with aggressive periodontitis who underwent SRP with additional PDT, unlike the group of patients who in addition to SRP were administered oral antibiotics (Skurska *et al.*, 2015). In her previous study neither the group of patient with chronic periodontitis who underwent SRP or SRP with additional ozone therapy, nor in the aggressive periodontitis patients treated with SRP with ozone therapy presented a statistically significant reduction of salivary MMP-8 levels after two months following treatment (Skurska *et al.*, 2010). In the control group of healthy or gingivitis patients, treatment had no significant influence on the GCF MMP-8 levels after two months, neither was oral hygiene instruction alone able to change the concentration of MMP-8 in saliva of chronic periodontitis patients after 7 months of observation (Marcaccini *et al*., 2010, Sexton *et al*., 2011). On the other hand, Kinney *et al.* observed an increase of salivary levels of MMP-8 in the healthy group after twelve months of observation with one prophylactic session performed at the sixth month, whereas in GCF this difference was not detectable (Kinney *et al*., 2011, Kinney *et al*., 2014). The periodontal treatment assisted by intraoral antibiotics showed significant reduction in MMP-8 levels; both when the classical combination of amoxicillin and metronidazole taken for 7 days was used, azithromycin for 3 days, or doxycycline in sub-antimicrobial dose for 2 years (Golub *et al*., 2008, Emingil *et al*., 2012, Goncalves *et al*., 2013, Skurska *et al*., 2015).

Depending on the kind of analysis and division into groups, Mäntylä *et al.* observed various results – when the whole cohort of the chronic periodontitis patients was analysed, the SRP treatment resulted in a substantial reduction of MMP-8; from 2177µg/L ± 2747 to 1339µg/L ± 1617 post-treatment (Mäntylä et al., 2006). It should be pointed out that the standard deviation at both time-points was bigger than the mean – in line with our data. The broad distribution of MMP-8 values between the patients and between the particular sites of the individual patient was noticed also by Kraft-Neumarker *et al* (Kraft-Neumarker *et al.*, 2012). These variations could be explained by the complex involvement of MMP-8 in the destructive but also in the protective anti-inflammatory role of MMP-8. Sampling is performed at a particular time-point and it cannot be assessed if the site at that particular moment is histologically in the phase of past or current inflammatory process or in the recovery phase. Interestingly, the non-smoker sites in the study of Mäntylä *et al.* presented higher levels of MMP-8 than sampled sites from smokers, and only in progressing non-smoker sites was the reduction of the enzyme levels after SRP statistically significant (Mäntylä et al., 2006). Hernandez *et al.* however found MMP-8 reduction after treatment in inactive sites, whereas in active sites the change was not significant (Hernandez *et al.*, 2010). It should be stressed that biomarker level indicating disease activity might be elevated for a short time only, and capturing that moment depends strictly on the timing of sampling. Based on analysed studies it becomes evident, that the differences in MMP-8 levels between particular studies varied by up to 100-fold, with pronounced inter-patient differences when using one laboratory method, and within similar patient profiles in single studies even by more than 7-fold (Kraft-Neumarker *et al.*, 2012). If we used the cut-off level of Mäntylä *et al.*, in our study, even after adjustment for laboratory dilution technique, our mean data from every time-point would still be below the 14.3 ng of aMMP-8/mL and thus they would all fall into the category of healthy levels (Mäntylä et al., 2003). Based on these findings it seems more realistic and rational to observe the changes in the MMP-8 levels in a specific patient, and to use them as an additional tool for individually tailored treatment plan for that patient, rather than looking for an objective ultimate cut-off value.

5.5.5 Prediction of the periodontal disease category using MMP-8 levels

Various researchers are looking for the ideal biomarker or group of biomarkers that could be used as a diagnostic tool instead of or in addition to the classical clinical

parameters. Optimal cut-off levels and optimal composition of various biomarkers have been tested to find ones with reliable sensitivity and specificity. Mancini *et al.* explored a screening test based on neutrophil collagenase activity and proposed a value of 80 nano-units of active MMP-8 as a threshold for the diagnosis of severe periodontitis (Mancini *et al.*, 1999). Gursoy *et al.* constructed a ROC for salivary MMP-8 levels and were able to discriminate periodontitis patients from the control group, especially when analysing the concentration with IFMA rather than ELISA (Gursoy *et al.*, 2010). Combining other salivary biomarkers, such as tissue inhibitor of matrix metalloproteinase (TIMP)-1, and pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP) with MMP-8, their AUC reached the value of up to 0.782. Interestingly, when only smokers were included in the analysis, the differences between the groups disappeared. In their subsequent publication Gursoy *et al.* showed that of numerous salivary biomarkers, only aMMP-8 with a threshold of 383.9 ng/mL had a sensitivity and specificity of over 0.5 to distinguish both generalised periodontitis and localised periodontitis patients from the control group (Gursoy *et al.*, 2013). GCF active MMP-8 levels measured by IFMA with a cut-off level of 754.1 ng/mL or by a dip-stick test set for 1 mg/L gave a sensitivity and specificity of between 0.83 and 0.96 to diagnose especially severe periodontitis-affected sites (Leppilahti et al., 2014a, Mäntylä et al., 2003). Higher diagnostic value from a range of analysed biomarkers corresponded however to myeloperoksidase (MPO). Ramseier *et al.* from a scope of analysed salivary biomarkers and pathogen biofilm ranked MMP-8 and osteoprotegerin (OPG) as the most important of a range of salivary biomarkers and pathogen biofilm for predicting patient disease category of healthy, gingivitis, mild chronic periodontitis or moderate to severe chronic periodontitis (Ramseier *et al.*, 2009). With a threshold of 87.0 ng/mL MMP-8 tested with classical ELISA from saliva, the odds ratio for a subject to be classified as a periodontitis patient was 5.3. However, when multiple biomarkers were combined, especially MMP-8, MMP-9 and OPG together with a red-complex anaerobic putative periodontal bacteria, such as *P. gingivalis and T.denticola,* the prediction of disease severity was much more accurate (AUC = 0.88, OR=24.6) . Miller *et al.* who used a threshold of elevated MMP-8 levels in saliva by more than two standard deviations from the mean obtained the odds ratio of more than 10 for more severe periodontal status based on clinical attachment loss or amount of deepened periodontal pockets. By combining both elevated MMP-8 and IL-1β levels they increased the odds ratio to identify a periodontally diagnosed patient to 45.5 (Miller *et*

al., 2006). When classifying a patient more comprehensively by the inflammatory burden, other authors were able to distinguish patients with a strong inflammatory burden from the healthy ones by measuring the aMMP-8 levels in oral rinse with the dentoELISA. IFMA was able to differentiate the two groups when adjusted for a number of teeth present, however, commercial ELISA did not show any differential capacity. However, the tests failed to distinguish patients with mild or moderate inflammatory burden from the healthy group. These findings indicate that there is a potential for rapid POC diagnostics allowing for a faster identification and screening of patients at risk for periodontal disease. However, given the complexity of periodontitis, it seems more appropriate to consider multiple biomarkers, reflecting distinct stages of periodontitis and different check-points of the disease such as infection, inflammation, immune dysregulation and bone resorption (Ramseier *et al.*, 2009). Up to now, there is no one universal biomarker even for the identification of a current periodontal disease.

5.5.6 Prediction of periodontal disease progression using MMP-8 levels

Correlation between clinical parameters and biomarkers of periodontal disease was investigated in numerous studies, the real benefit in daily clinical practice would be however a biomarker-based test that could be used as a diagnostic and risk-assessment tool for future tissue breakdown, thus giving the practitioner time to act before irreversible damage occurs. In our study we tried to correlate the levels of active MMP-8 with the changes of the most commonly used clinical parameters, that is, pocket depth and clinical attachment level occurring between the given time-point and a consecutive visit, that is, two to four months later. We were testing any detectable change, that is, change over 0.2 mm, as that was the accuracy of the electronic periodontal probe used in the study or a change over 0.5 mm, as that is a limit of what can be clinically detected using manual periodontal probe in standard clinical settings. Lee *et al.* measured active and latent forms of the collagenase separately and concluded that active collagenase pooled from 6 sites per subject was 5 times higher in the patient group with progressive loss of connective tissue compared to patient group with gingivitis only. Additionally, in 8 out of 14 progressive patients a large increase of collagenase activity was observed one to two months before clinically detectable loss of attachment, and in the remaining 6 patients it was simultaneous with worsened clinical parameters (Lee *et al.*, 1995). Some authors noticed persistently elevated levels of MMP-8 despite undergoing SRP with or without periodontal surgery – those patients,

especially smokers, showed poor response to therapy and could be identified as subjects at risk of further tissue destruction (Mancini et al., 1999, Mäntylä et al., 2006). As already mentioned, finding an universal MMP-8 cut-off level for all patients seems elusive and so in a series of studies, some researchers grouped patients in clusters or concentrated on the change of the level of biomarkers rather than on absolute values. Reinhardt *et al.* reported that postmenopausal females who experienced an increase of over 0.08 total MMP-8 scanning units in the examined sites during the first year of periodontal maintenance had 50% more chance of more progressive relative attachment loss at the end of the second year of treatment. This association was influenced primarily by patients on placebo; when the groups were analysed separately, those treated with SDD showed no statistically significant results (Reinhardt *et al.*, 2010). At the same time, in line with our own data, the absolute MMP-8 values at baseline, similarly like our data, showed no association with change of attachment level occurring at the subsequent visits. From the range of biomarkers tested in GCF, increase of IL-1β levels was ranked higher than MMP-8 with respect to the ability to distinguish patients who would develop attachment loss. Sexton performed a ROC to evaluate the change in salivary MMP-8 concentration with respect to the response to therapy (Sexton *et al.*, 2011). He defined a group of responders that were patients who improved in four clinical categories by at least 20% at both follow-up visits, and for that group the AUC for the change of MMP-8 levels was the highest, followed by OPG, demonstrating positive response to the therapy. Leppilahti *et al.,* based on constructed contingency tables and ROC curves, concluded that in smokers baseline GCF aMMP-8 levels >770 ng/mL indicated a 22-percentage-point risk increase of poor treatment outcome defined as attachment loss (Leppilahti *et al.*, 2014b). In turn, sites with levels <160 ng/mL had a decreased risk for weak treatment outcome by 25 percentage-points, that is reattachment of the junctional epithelium could be expected. In non-smokers this prediction was not possible due to the lack of statistical significance. In their following study, a group of researchers around the same author combined the data from four separate longitudinal studies (Leppilahti *et al.*, 2015). The optimal cut-off levels for the GCF MMP-8 levels were presented on a scale from 0 to 1 and can be regarded as percentage of the population maximum. Separate threshold were calculated for non-smokers and smokers sites; double-positive test results marked a 46 and 39 percent-point risk increase for the compromised outcome that is CAL gain of less than 2mm, respectively. The differences between physiologic and pathologic MMP-8 levels at

individual sites differed remarkably between various patient patterns, especially among non-smokers, which is a common observation in studies dealing with MMP-8. Other clusters were built by Kinney *et al.* who observed that 71% of subjects with high salivary biomarkers and biofilm pathogens showed periodontal disease progression defined by at least two sites demonstrating more than 2 mm of CAL loss within 6 months (Kinney *et al.*, 2011). On the other hand, 76% of those with low biofilm and biomarker levels were periodontally stable. In a follow-up study Kinney *et al.* determined a statistically significant difference in baseline median GCF MMP-8 levels for the patients who were six months later classified as stable or progressive patients; 9,328 pg/mL (range 4,695–26,697) versus 10,931 pg/mL (range 4,610–23,772), respectively (Kinney *et al.*, 2014). However, when establishing the ranking order of the analysed biomarkers, IL-1β had the strongest value in the prediction of periodontal progression, followed by OPG, MMP-8 and then MMP-9. The authors analysed a wide spectrum of parameters, including salivary, serum, GCF biomarkers, clinical measurements, and biofilm profiles, and suggested that the greatest sensitivity was achieved when salivary biomarkers were used and best specificity was observed when GCF biomarkers were adopted to identify periodontal disease progression. A disproportionate low sensitivity and high specificity, 23% and 95% respectively, were observed when only GCF biomarkers were considered.

Summing up, various methods were used to find an optimal, universal biomarker and it's cut-off level to predict periodontal disease progression. Until now, there seems to exist no single biomarker, neither biofilm nor clinical parameter, which could be used in a predictable way in a heterogenic group of patients to fulfil this wish of the dental community.

6. Conclusions

Classic diagnostic methods in periodontology are able to detect only ongoing inflammatory process or the extent of already lost tissues resulting from previous destructive inflammatory cascades. In this study we verified the usefulness of GCF aMMP-8 levels as a diagnostic tool during periodontal maintenance therapy. Firstly, we evaluated if the selected biomarker, aMMP-8, corresponded with the gold standard methods to detect the patients and sites at risk. In our study population the levels of the enzyme moderately correlated with PD at the initial visit, but not at following maintenance visits. Active MMP-8 levels were clearly higher at sites demonstrating

positive BOP than at sites without this clinical indicator of ongoing inflammation, except for the last visit, where the differences were not statistically significant. Periodontal treatment consisting of scaling and root planing with or without adjunct antibiotics reduced the levels of aMMP-8 on consecutive visits following initial periodontal therapy; however, large variations were observed between particular patients and sites. Clinically most relevant was the evaluation of the prognostic ability of the enzyme to detect future tissue breakdown. Regardless of the definition of disease progression, whether based on the change of PD levels or CAL loss, at no point in time did the aMMP-8 levels measured in four GCF samples per patient predict progression or relapse of periodontal disease at patient level at a follow-up visit.

In conclusion, currently available biomarker tests based on MMP-8 levels provide us with a predefined, absolute MMP-8 cut-off value which can hardly be used in the whole range of patients who present in the dental office. Problem arises from the heterogeneity of the patients, their general health condition, medications, smoking habits or hormonal status influence immune response to the pathogens. The same level of biomarker can be compatible with health in one patient and pathology in another one due to individual variability. It is the nature of periodontitis that it progresses episodically, infrequently and slowly in most chronic periodontitis patients, with often only a small number of sites experiencing attachment loss simultaneously. It makes it almost a Russian roulette for the professional health provider to choose the most adequate sampling sites with perfect timing, that is, at the peak of their collagenolytic activity. Further studies, evaluating a wider range of biomarkers, targeting different check-points of periodontal disease process and possibly combined with plaque pathogens would be required to develop a method of earlier and more reliable detection of patients with risk of periodontal disease progression.

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„Ich, Marta Czownicka, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema:
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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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