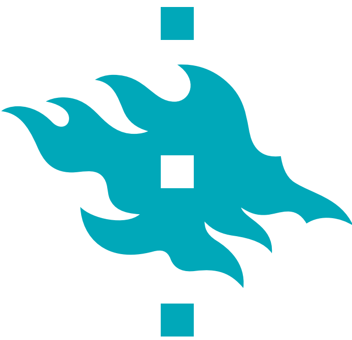

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SAEED ALASSIRI

UTILITY OF ACTIVE MMP-8 AS A TEST BIOMARKER IN PERIODONTAL AND PERI-IMPLANT DISEASES: DIAGNOSIS, PREVENTION AND TREATMENT OUTCOMES

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CLINICUM
DEPARTMENT OF MEDICINE
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UNIVERSITY OF HELSINKI

UTILITY OF ACTIVE MMP-8 AS A TEST BIOMARKER IN PERIODONTAL AND PERI-IMPLANT DISEASES: DIAGNOSIS, PREVENTION AND TREATMENT OUTCOMES

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I dedicate this thesis to my lovely family,
my wife Sarah and my son Jassar,
and to my supervisors.
I couldn't done this without you.
Thank you for all of your support along the way.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are here by Roman numerals (I – V).

I Silbereisen A, Alassiri S, Bao K, Grossmann J, Nanni P, Fernandez C, Tervahartiala T, Nascimento GG, Belibasakis GN, Heikkinen AM, Lopez R, Sorsa T, Bostanci N. Label-Free Quantitative Proteomics versus Antibody-Based Assays to Measure Neutrophil-Derived Enzymes in Saliva. Proteomics Clin Appl. 2019 Dec doi: 10.1002/prca.201900050.

II Alassiri S, Parnanen P, Rathnayake N, Johannsen G, Heikkinen AM, Lazzara R, van der Schoor P, van der Schoor JG, Tervahartiala T, Gieselmann D, Sorsa T. The Ability of Quantitative, Specific, and Sensitive Point-of-Care/Chair-Side Oral Fluid Immunotests for aMMP-8 to Detect Periodontal and Peri-Implant Diseases. Dis Markers. 2018 Aug doi: 10.1155/2018/1306396.

III Sorsa T, Alassiri S, Grigoriadis A, Räisänen IT, Pärnänen P, Nwhator SO, Gieselmann DR, Sakellari D. Active MMP-8 (aMMP-8) as a Grading and Staging Biomarker in the Periodontitis Classification. Diagnostics (Basel). 2020 Jan, doi: 10.3390/diagnostics10020061.

IV Öztürk VÖ, Emingil G, Umeizudike K, Tervahartiala T, Gieselmann DR, Maier K, Köse T, Sorsa T, Alassiri S. Evaluation of active matrix metalloproteinase-8 (aMMP-8) chair-side test as a diagnostic biomarker in the staging of periodontal diseases. Arch Oral Biol. 2021 Jan 20;124:104955. doi: 10.1016/j.archoralbio.2020.104955.

V Emingil G, Gürkan A, Tervahartiala T, Hernandez M, Özgül S, Sorsa T, Alassiri S. Adjunctive Effects of a Sub-Antimicrobial Dose of Doxycycline on Clinical Parameters and Potential Biomarkers of Periodontal Tissue Catabolism. Dent J (Basel). 2019 Jan, doi: 10.3390/dj7010009.

ABBREVIATIONS

aMMP-8	Active matrix metalloproteinase 8
ABL	Alveolar bone loss
AL	Attachment loss
APC	Antigen-presenting cells
AST	Aspartate aminotransferase)
BM	Basement membrane
BOP	Bleeding on probing
CAL	Clinical attachment loss
CRP	C-reactive protein
CTLP	Chymotrypsin-like proteinase, dentilisin
DAMP	Danger-associated molecular pattern
DTPA	Diethylenetriaminepentaacetic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E-selectin	Endothelial leukocyte adhesion molecule
GCF	Gingival crevicular fluid
HMT	Host modulation therapy
IL	Interleukin
ICAM-1	Intercellular adhesion molecule 1
IFMA	Time-resolved immunofluorometric assay
Ig	Immunoglobulin
JE	Junctional epithelium
LPS	Lipopolysaccharides
L/SDD	Low dose doxycycline/ Subantimicrobial-dose doxycycline
MGI	Modified gingival index
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
MT-MMPs	Membrane-type matrix metalloproteinases
NF-kB	Nuclear factor kappa B
OPG	Osteoprotegrin
PAMP	Pathogen-associated molecular pattern
PGE	Prostaglandin E
PBI	Papillae bleeding index
PPD/PD	Probing/Pocket depth
PRR	Pattern recognition receptor
PI/VPI	Plaque index/visible plaque index
PISF	Peri-implant sulcular fluid
PoC	Point-of-care
PMN	Polymorphonuclear leukocyte (neutrophil)

PD	Pocket depth
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
SD	Standard deviation
SDD	Sub-antimicrobial doses of doxycycline
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamid gel electrophrasis
SNP	Single nucleotide polymorphism
TC	Tetracycline
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TRAP	Tartrate-resistant acid phosphatase
TQHPI	Modified Guigley and Hein plaque index

ABSTRACT

Active matrix metalloproteinase (aMMP-8), collagenase-2, is a key mediator in the destruction of tissues seen in periodontitis and peri-implantitis. It can be detected in oral fluid samples, including gingival crevicular fluid (GCF), saliva, mouth rinse and peri-implant sulcular fluid (PISF). Active MMP-8, which is mainly expressed by neutrophils, can degrade almost all extracellular matrix and basement membrane components; it is causes particularly high levels of degradation in type I collagen, which is present in periodontal ligaments.

In the first part of this thesis, we demonstrated that during an experimental gingivitis study aMMP-8 was not detectable in saliva. Therefore, this study confirms the evidence; aMMP-8 is detected in periodontitis. Simultaneously, the lateral-flow chairside aMMP-8 immunotests, PerioSafe and ImplantSafe with the Oralyzer reader, which are based on Professor Timo Sorsa's research, were introduced and validated to analyse the levels of aMMP-8 in mouth rinse or PISF. Thus, the last parts of the thesis evaluate the accuracy and reliability of these developed tools: PerioSafe and ImplantSafe/Oralyzer, and whether they could provide an easy-to-use and quick way of identifying periodontitis and peri-implantitis.

Our results indicated that the tools were successful in their detection. They were also very accurate in terms of both sensitivity and specificity with clinical periodontal parameters, such as bleeding on probing (BOP) and plaque index (PI). In addition, study V has investigated the effectiveness of sub-antimicrobial doses of doxycycline (SDD) treatment when combined with non-surgical periodontal therapy, and their effect on potential GCF biomarkers in periodontal tissue destruction over a period of twelve months. The analysed biomarkers were MMP-8, -9, -13, myeloperoxidase (MPO), osteoprotegerin (OPG), and tartrate-resistant acid phosphatase 5 (TRAP-5), which were determined by various immunofluorometric analyses (IFMA) and enzyme-linked immunosorbent assays (ELISAs). The results demonstrated reduced GCF levels in two MMPs that most commonly associated with periodontitis; MMP-8 and -9, and MPO, which potentially causes oxidative activation in them. However, MMP-13 and TRAP-5 could be detected in the relevant GCF samples, whereas the OPG levels also decreased significantly as a result of adjunctive SDD therapy.

This thesis showed the reliability of these tools, PerioSafe and ImplantSafe/Oralyzer, for the successful detection, prediction and monitoring of the progress of periodontitis and peri-implantitis, and their response to treatment. Additionally, we delved further in to the beneficial effects of sub-antimicrobial doses of doxycycline therapy.

1. INTRODUCTION

Periodontitis and peri-implantitis are common across the globe. They are inflammatory disorders that affect the teeth and dental implants. They are caused by infection, and both actively degrade and destroy periodontal, peri-implant soft and hard tissues. Periodontal and peri-implant tissues are mainly made up of type I collagen. The proteolytic enzyme, which is known to cause the majority of active periodontal/peri-implant soft and hard tissue degeneration (APD), is MMP-8, also known as neutrophil collagenase or collagenase-2. MMP-8 belongs to the MMP family. Ca^{2+} and Zn^{2+} -dependent endopeptidases are genetically different MMPs but they have a similar structure. They are capable of degrading most extracellular matrix and basement membrane protein components in both the physiologic repair and pathologic destruction of tissues, such as healing wounds, tissue remodeling and embryonic development (Kinane et al. 2000, Nagase et al. 2006).

In the MMP family, MMP-8 is part of the interstitial collagenase subgroup. Whereas α2-macroglobulin and TIMPs regulate MMP activity. When levels of MMPs and TIMPs in tissues are imbalanced, this can lead to irreversible periodontal and peri-implant destructive pathology involving soft and hard tissue degeneration. Periodontopathogenic bacteria and their virulence factors, in combination with host-derived proinflammatory mediators, trigger degranulation. Such degranulation leads to MMP-8 being released from neutrophils (Sorsa et al. 2006, 2016, Bernasconi et al. 2015). Links have recently been made between elevated MMP-8 levels in oral fluids, particularly the active form of MMP-8 (aMMP-8), and periodontal and peri-implant inflammation disorders (Sorsa et al. 1988, Cox et al. 2006, Al-Majid et al. 2018). Levels of active MMP-8 are directly associated with progressive periodontal and peri-implant disease activity, and they can therefore be used to predict the development and progression of these diseases. Positive correlations have been found between elevated levels of aMMP-8 in oral fluids, such as gingival crevicular fluid (GCF), mouth rinse, saliva and peri-implant sulcular fluid (PISF), and clinical periodontal parameters, such as bleeding on probing (BOP), probing pocket depth (PPD, which is in this work abbreviated as PD) and clinical attachment loss (CAL). When periodontal and peri-implant treatments are used successfully, aMMP-8 levels can be decreased (Hernandez et al. 2010, Thierbach et al. 2016, Mauramo et al. 2018).

Periodontitis can be treated in a simple and inexpensive way if it is diagnosed at an early development stage. This can be challenging since the disease tends to be asymptomatic. Therefore, highly trained professionals and specific equipment are needed for a diagnosis purposes. and these are not always available in areas that are poor in resources (Mohd-Dom et al. 2014, 2016). Recently, the aMMP-8 PerioSafe and ImplantSafe tests were introduced; they utilise an Oralyzer reader, which provides quantitative results, and they are used to diagnose periodontitis and peri-implantitis. The tests, which are similar to pregnancy tests, are easy to use and can be carried out at the point-of-care. Studies have already confirmed

the accuracy of their diagnoses. The tests measure aMMP-8 levels in oral fluid with a lateral flow immunoassay, and the results can be used to assess the inflammatory burden of the periodontium (Sorsa et al. 2016, Heikkinen et al. 2016, Raisanen et al. 2019).

Liquid chromatography mass spectrometry (LC-MS) -based proteomic approaches have proven valuable for assessing proteins in the saliva of both healthy and diseased patients. Although over 5,000 proteins have been identified in saliva, it is difficult to further characterise them as there are not enough antibodies available with high specificity for all of these proteins. Furthermore, none of these approaches have been applied in the clinical diagnosis of periodontitis. For this reason, antibody-based immunoassays, which employ antibodies that target a specific protein, are the main method for the quantification of proteins (Grassi et al. 2016, Bostanci et al. 2017).

In inflammatory diseases, such as periodontitis, it is important to be able to modulate the inflammatory responses and manage the outcomes. Although some inflammatory mechanisms aim to protect, they can actually result in self-damage if the inflammation is not resolved sufficiently. The sub-antimicrobial doses of doxycycline (SDD) therapy can be used effectively to manage periodontitis when combined with non-surgical therapy because it can regulate the host response (Golub et al. 2020, Sorsa et al. 2020). Numerous studies have demonstrated that SDD can affect the production of MMP-8 and collagen metabolism, thus preventing bone loss and the progression of periodontitis. It is believed that MMPs play a role in the periodontal tissue destruction during the inflammatory process, in which cytokine and chemokine networks, and the host's immune response are heavily involved (Golub et al. 2020, Sorsa et al. 2020).

2. LITERATURE REVIEW

2.1 MATRIX METALLOPROTEINASE 8 (MMP-8)

2.1.1 General characteristics of MMPs

Human matrix metalloproteinases (MMPs) are a family of enzymes that are structurally, but not genetically, similar. The group consists of 23 calcium-dependent and zinc-dependent endopeptidases (Nagase et al. 2006, Jackson et al. 2010, Butler et al. 2013, Checchi et al. 2020). MMPs play a crucial role in tissue remodelling, development and homeostasis and they regulate a variety of functions such as cell differentiation, migration and apoptosis (Nyberg et al. 2006, Hannas et al. 2007). In addition, they are capable of destroying most extracellular matrix (ECM) and basement membrane (BM) protein components. Connections have been made between a large number of inflammatory disorders and MMPs (Nagase et al. 2006, Van Lint et al. 2006, Morais et al. 2017). There is plenty of evidence to support the fact that MMP upregulation and downregulation occurs as a response to inflammatory mediators, such as cytokines and chemokines (Franco et al. 2017, Roomi et al. 2017). Activated MMPs can also activate other MMPs (Vu and Werb 2000, Page-McCaw et al. 2007). In most cases, MMPs are synthesised and secreted as proenzymes. Latent, proMMPs can be activated in three ways: (1) intracellularly, (2) at the cell surface by membrane-type (MT-) MMPs, and (3) extracellularly by other proteases (Visse and Nagase 2003, Holopainen et al. 2003). Other MMP activators include serine proteinase, such as plasmin and trypsin (particularly human trypsin-2) (Sorsa et al. 1997, Visse and Nagase 2003, Bjorklund and Koivunen, 2005). The initial activation of serine proteinase in the proMMP activation cascade is thought to occur with human trypsin-2 (Sorsa et al. 1997). Human trypsin-2 is also responsible for the direct degrading of native interstitial collagen types I and II (Stenman et al. 2005).

Cysteine proteinases released from gingival fibroblasts and proforms of serine proteinases can result in proMMP activation (Cox et al. 2006). ProMMPs can be activated by reactive oxygen species (ROS) as well as bacterial and candidal proteinases (Saari et al. 1990, Sorsa et al. 1992). The typical uniformity of different activation pathways is that the molecular weight of proMMPs changes when the N-terminal propeptide is cleaved and removed, and the active forms are usually 10 – 20 kDa less than the proforms (Nagase et al. 2006, Page-McCaw et al. 2007). When physiological conditions are normal, there is a balance between active and inactive MMPs. If this balance is disrupted, excess tissue destruction and pathological conditions, such as periodontitis, can occur (Mantyla et al. 2006, Sorsa et al. 2006, Visser and Ellen 2011).

MMPs are classified into six protease groups based on substrate specificity, domain structure and cellular localization: collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10), matrilysins (MMP-7, MMP-26

and MMP-11), membrane-type (MT-) MMPs (MMP-14, MMP-15, MMP-16, MMP-17 MMP-24 and MMP-25) and others MMPs (Stocker et al. 1995, Nagase et al. 2006).

MMPs have a common domain structure, which consists of a pro-domain, catalytic domain and hemopexin domain. There are 80 amino acids in the pro-domain, around 170 in the catalytic domain and about 200 in the hemopexin domain. The pro-domain has a highly conserved cysteine residues, which are able to bind to the zinc ion in the catalytic domain (Nagase et al. 2006). Activation occurs when the pro domain is removed, and the catalytic site is revealed and prepared for enzymatic activity. A linker peptide (the hinge region) acts as a contact point between the hemopexin domain and the C-terminal domain (Nagase et al. 2006, Page-McCaw et al. 2007).

There are three main levels of MMPs regulation; gene expression, proenzyme activation and endogenous inhibition (Overall et al. 2002, Sorsa et al. 2016). MMPs can be inhibited by specific tissue inhibitors of metalloproteinase (TIMP-1, -2, -3, and -4), serine proteinase inhibitors, and non-specific endogenous inhibitors, such as α2-macroglobulin (Herman et al. 2001, Yabluchanski et al. 2013). In addition, low-dose-doxycycline/subantimicrobial-doses of doxycycline (L/SDD) therapy can inhibit MMPs (Golub et al. 1995, 2020).

2.1.2 Expression and activity of MMP-8

MMP-8 (neutrophil collagenase) is synthesised in the bone marrow during the myelocyte stage of neutrophil development. It is stored in specific granules (Hanemaaijer et al. 1997). There are also other mesenchymal cell sources that express and secrete MMP-8 in smaller quantities that are less glycosylated than the polymorphonuclear leukocytes (PMNs) (Sorsa et al. 1997, Hanemaaijer et al. 1997). Non-PMN lineage cells include epithelial cells, fibroblasts, osteoblasts, odontoblasts and endothelial cells (Tervahartiala et al. 2000, Palosaari et al. 2000, Wahlgren et al. 2001, Owen et al. 2004).

Periodontopathogenic bacteria as well as their virulence factors, such as microbial-derived proteases, can cause stimulation of neutrophil degranulation during phagocytosis. The microbial-derived proteases generally include gingipains, trypsin-like cysteine proteinases of *Porphyromonas gingivalis* (*P. gingivalis*), and dentilisin (*Td*-CTLP), a chymotrypsin-like proteinase of *Treponema denticola* (*T. denticola*) (Imamura et al. 2003, Gursoy et al. 2018). Neutrophil degranulation is also triggered by prostaglandins and cytokines, which include host-derived inflammatory mediators, such as the tumour necrosis factor (TNF-α) and interlukine (IL-1β) (Ryan et al. 1996, Sorsa et al. 1992, 2004). Subsequently, neutrophil degranulation leads to the release of latent MMP-8 from specific granules and MPO from the primary granule (Saari et al. 1990, Tervahartiala et al. 2000, Leppilahti et al. 2014). The conversion of the potent oxidant, hypochlorous acid (HOCl), is catalysed by myeloperoxidase (MPO). This not only involves antimicrobial activity, but it also modifies the balance between proteases/anti-proteases by playing a significant role in

how connective tissue catabolism and degradation are regulated (Wang et al. 2007, Khan et al. 2018). Depending on the oxidant/enzyme molar ratio, HOCl can quickly activate proMMP-8 and proMMP-9 (Weiss et al. 1985, Peppin et al. 1986, Saari et al. 1990, Sorsa et al. 2006). MPO in the neutrophils' primary granules can participate in MMP-8 activation, which supports the oxidative role of MPO in the activation of latent MMP-8 and MMP-9. Similarly, the inactivation of TIMP-1 is based on evidence concerning progressive chronic periodontitis in humans (Hernandez et. al 2006, Marcaccini et al. 2010). Furthermore, MMP-8 and MPO show a highly specific site-diagnosis of chronic periodontitis. All in all, these findings indicate that MPO enhances MMP-8 activation during the progression of periodontal disease (Leppilahti et al. 2014). The known ECM substrates of MMP-8 are collagens types I, III, VII, X, gelatine, fibrinogen, bradykinin and angiotensin, as well as pro-inflammatory and anti-inflammatory cytokines/mediators. In addition to this, MMP-8 can inactivate the α2-macroglobulin and α1-proteinase inhibitors (Hasty et al. 1987, Diekmann et al. 1994, Hiller et al. 2000, Sternlicht and Werb 2001).

Collagen type I is the main substrate of MMP-8. The ECM protein in several different tissue types, such as periodontium, is mainly structured by this collagen. MMP-8 remodels and degrades the vascular basement membrane and ECM, allowing leukocytes to transmigrate into the inflammatory site. Furthermore, MMP-8 also degrades several non-ECM substrates, such as chemokines, and protease inhibitors (Van Lint and Libert 2006). Neutrophils play an important role in the innate immunity system and removing pathogens. The movement and chemotaxis of neutrophils rely on MMP-8 being released (Nauseef 2007, Tester et al. 2007). However, excessive damage and degradation of tissues can lead to destructive inflammation, which in turn is caused by neutrophil degranulation, and the release and activation of MMP-8. Therefore, MMP-8 contributes significantly in a number of inflammatory disorders that lead to the destruction of tissue, such as periodontitis (Herr et al. 2007, Sorsa et al. 2016, Morais et al. 2018). MMP-8 is also associated with the cancer progression and the healing of wounds (Dejonckheere et al. 2011, Rohani and Parks 2015). MMP-8 in oral fluid can be found in different molecular forms, wherein the complex form is >110 kDa, the latent and active neutrophilic form is 85-65 kDa, the latent and active mesenchymal-type collagenase form is 65-45 kDa and fragments are 30-20 kDa (Hanemaaijer et al. 1997, Geijersstam et al. 2005, Gursoy et al. 2018).

2.1.3 Genetics of MMP-8

The MMP-8 gene is situated in the cluster of MMP genes in 11q22.3 (Hasty et al. 1990, Pendás et al. 1996). The gene's activity is regulated by three single-nucleotide polymorphisms (SNPs) in the promoter region of MMP-8, −799C/T (rs11225395), −381A/G (rs1320632), and +17C/G (rs2155052) (Wang et al. 2004). According to Heikkinen et al. (2016), MMP-3 and vitamin D receptor genetic polymorphisms were reported to be associated with initial periodontitis in Finnish adolescents. In contrast, there was no association between SNPs and aMMP-8. However, further research needs to be carried out into

how these polymorphisms affect MMP-8 tissue concentrations (Aquilant et al. 2007, Pradhan-Palikhe et al. 2012).

2.2 OTHER PROTEINASES AND THEIR INHIBITORS IN PERIODONTAL TISSUE DESTRUCTION

2.2.1 Matrix metalloproteinase 13 (MMP-13)

The expression of MMP-13 (collagenase-3) is very restricted but it has a broad substrate specificity. It is an efficient catalyst enzyme (Knauper et al. 1996, Ala-aho et al. 2005, Checchi et al. 2020). MMP-13 was first found to be expressed in breast carcinomas (Freije et al. 1994), and later, it has been identified in human periodontitis (Uitto et al. 1998). This suggests that it facilitates the conversion of pocket epithelium into periodontal connective tissue through the lamina propria stromal cells of the gingival sulcus, such as macrophage and fibroblasts (Uitto et al. 1998, Tervahartiala et al. 2000, Kiili et al. 2002). MMP-13 is a very efficient degrader of collagen type II – it is ten times more efficient than other collagenases (Knauper et al. 1996). MMP-13 is secreted as a 60 kDa glycoprotein, which is converted to 48 kDa during proteolytic activation (Moilanen et al. 2003, Virtanen et al. 2017). GCF samples were taken from periodontitis patients before they underwent treatment, and immunoblotting assays for MMP-13 revealed complex, proenzyme and activated enzyme forms. Samples that were taken after scaling and root planing (SRP) contained significantly less band absorbance (Golub et al. 1997, Kiili et al. 2002, Ilgenli et al. 2006). According to a study carried out by Ejeil et al. (2003), which investigated the possible correlation between the presence of enzymes, such as MMP-1, -2, -9, and -13, and collagen loss, GCF from patients with periodontitis contained proenzyme, active and fragmented forms of these enzymes. When they were present in human gingival explants, collagen fibres decreased. The conclusion made by the authors was that there was a link between these metalloproteinases and periodontitis-related ECM degradation.

2.2.2 Matrix metalloproteinase 9 (MMP-9)

It has been suggested that MMP-9 (gelatinase-B) plays a role in the destruction of periodontal tissue. Patients with periodontitis have been found to have elevated levels in their GCF, but these declined with conventional periodontal treatment (Makela et al. 1994, Ingman et al. 1996, Westerlund et al. 1996, Beklen et al. 2006). The degradation of a number of proteins is also attributed to gelatinases: collagen types IV, V, VII, X and XI; elastin; and the components of basement membrane (Owen and Campbell 1999, Beklen et al. 2006).

MMP-9 is most effective in degrading aggrecan, fibronectin, fibrillin and collagen types I, II, III, V, VI and X (Konttinen et al. 1998, Kahari and Saarialho-Kere 1999). In periodontitis, neutrophils are the principle source of MMP-9. Macrophages also produce MMP-9 but to a lesser extent (Westerlund et al. 1996, Pirila et al. 2001). MMP-9 appears to be the principle gelatinase present in the gingival tissue, saliva, dental plaque and GCF of chronic periodontitis patients (Sorsa et al. 1995, Golub et al. 1995, Vandooren et al. 2013).

2.2.3 Myeloperoxidase (MPO)

MPO is a lysosomal enzyme and can be considered to be a biomarker of inflammation and oxidative stress. It is mainly produced by neutrophils (PMNs) (Khan et al. 2018) and monocytes (Podrez et al. 2000). Its gene is located on chromosome 17 (Khan et al. 2018) and when active, its molecular weight is 150 kDa. The primary granules of PMNs store MPO (Cao et al. 1989, Borregaard et al. 2007). MPO is released into extracellular fluid through inflammatory mediators and oxidative stress. It has an antibacterial effect due to the secretion of H_2O_2 (Khan et al. 2018). MPO plays an essential role in oxidatively activating proMMP-8 and proMMP-9, and deactivating TIMP-1 (Saari et al. 1990). In addition, increased levels of MPO in oral fluid were detected in patients with periodontitis and peri-implantitis (Liskmann et al. 2004, Gursoy et al. 2013, Nizam et al. 2014). The oxidative effect of MPO is described in further detail in Section 2.1.2.

2.2.4 Neutrophil elastase (PMN elastase)

Neutrophil elastase is a serine proteinase and it is secreted by neutrophils' azurophilic granules during phagocytosis stimulation and cell lysis. In the same way as other serine proteinases, elastase has broad substrate specificity and it can degrade a range of tissue molecules, such as elastin, fibronectin, collagens, proteoglycan and laminins. It is thought to possibly have some direct anti-microbial characteristics. Increased levels of PMN elastase in different oral fluids, such as GCF and saliva, have been associated with periodontitis and peri-implantitis (Ingman et al. 1996, Nizam et al. 2014, Bujanda et al. 2019).

2.2.5 Tartrate-resistant acid phosphatase 5 (TRAP-5)

Tartrate-resistant acid phosphatase is a purple acid phosphatase that contains iron. Its expression is via osteoclasts and other monocyte–derived macrophage cells (Halleen et al. 2003). When bone resorption occurs, TRAP-5 (or only TRAP) is secreted from osteoclasts and is indicative of the bone resorption rate. Research is currently being conducted into TRAP as a blood-borne biochemical marker for bone resorption and osteoclast activity. A correlation has been observed between TRAP serum concentrations and bone resorption rates. The administration of osteoprotegerin (OPG) reduces the amount of TRAP serum and osteoclastic bone resorption, which is a characterising feature of periodontitis progression (Halleen et al. 2003, Kirstein et al. 2006, Hienze et al. 2015).

2.2.6 Osteoprotegerin (OPG)

Osteoprotegerin is secreted by osteoblasts and several other types of cells. Many of the factors that regulate osteoblasts' expression of receptor activator of nuclear factor kappa-B ligand (RANKL) also regulate the expression of OPG. Previous studies have not always been in agreement but generally it is accepted that if the up-regulation of RANKL expression occurs, OPG expression is down-regulated, or at least it is induced less than RANKL. Therefore, changes in the RANKL/OPG ratio favour osteoclastogenesis (Bostanci

et al. 2007, Hienze et al. 2015). The activity and numbers of osteoclasts may increase if the RANKL/OPG ratio changes due to either an increase in osteoclasts or a reduction in the RANKL/OPG ratio, or if both change in a way that alters the ratio to favour RANKL (Bostanci et al. 2007, Hienze et al. 2015).

RANKL is a homotrimeric protein. It can be secreted by activated T-cells and is usually bound to the membranes of osteoblastic and activated T-cells. Alternative splicing or proteolytic cleavage lead to the membrane form of the secreted protein (Crotti et al. 2003, Jin et al. 2007, Silva et al. 2015).

Receptor activator of nuclear factor kappa-B (RANK) is a homotrimeric transmembrane protein belonging to the TNF receptor superfamily. In dendritic cells and mature osteoclasts, and at a protein level, its expression is lower than that of RANKL. It is expressed in mammary glands and certain types of cancer cells, such as prostate and breast cancer. If the concentrations of OPG are relatively high compared to the expression of RANKL, OPG binds with RANKL and prevents it from binding with RANK. This leads to a decreased number of osteoclasts being formed and apoptosis of existing osteoclasts (Mogi et al. 2004, Hienze et al. 2015).

2.2.7 Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs exert extracellular control over interstitial collagenases and other MMPs. Several different cells express TIMPs, particularly TIMP-1, such as monocytes/macrophages, host fibroblasts, osteoblasts, keratinocytes and endothelial cells (Meikle et al. 1994, Rayan and Golub 2000). TIMPs have also been observed in GCF. TIMPs play a role in regulating ECM metabolism, but their principal function is seen to be the inhibition of MMPs (Kahari et al. 1999, Folgures et al. 2004). The balance between MMPs and TIMPs is essential in controlling ECM degradation (Nagase et al. 2006). There are four members of the TIMP family: TIMPs 1–4. They present distinct expression profiles and biochemical characteristics (Folgures et al. 2004). TIMP-1 is a 28 kDa glycoprotein, which has the ability to inhibit fast all MMPs, particularly MMP-1 (Nomura et al. 1993, Kubota et al. 1996). In GCF, higher TIMP-1 levels have been observed in periodontitis sites compared to healthy sites (Haerian et al. 1995, Choi et al. 2004). Furthermore, the salivary TIMP-1 levels are significantly decreased following periodontal treatment (Hayakawa et al. 1994, Gursoy et al. 2010).

2.3 CLASSIFICATION AND DIAGNOSIS OF PERIODONTAL DISEASES

Periodontitis is widespread; 10-15% of adults across the world suffer from it (Petersen and Ogawa 2012). The percentage is much higher in the United States, where 46% of adults have periodontitis. Among these individuals, 8.9% suffer from severe periodontitis, which can severely affect their quality of life because it can lead to teeth loss and mastication difficulties. (Eke et al. 2015).

A number of clinical indices and measurements are applied in the diagnosis of periodontal diseases. These include BOP, PD, radiographs and CAL level assessments. Records are also made on the amount of plaque and any relevant medical and hereditary factors, such as certain systemic diseases. The symptoms of periodontitis include halitosis, bleeding, swelling and redness of the gingiva, gingival recession, or instability of teeth. A periodontal probe is used to measure the depth of periodontal pockets and to assist with a clinical diagnosis of periodontitis. If the pocket depth is 4 mm or more, the diagnosis is termed pathological. Radiographs are used to assess how much alveolar bone has been lost around the teeth, as this is a sign tissue destruction caused by episodes of periodontitis. There are often no symptoms until the teeth loosen, and the worst outcome is the loss of the affected teeth. The risk of this occurring is significantly greater if the periodontal pockets are 7 mm deep or more (Khan et al. 2015).

One definition for diagnosis is to use signs and symptoms to identify a disease, while classification is defined as the process of dividing into groups. A clinician's diagnosis can be considered as an initial 'best assumption' concerning a patient's state of health. The first periodontal classification system was introduced in 1989. The system had some significant issues; there was no clear definition between the various diagnostic categories or a standard foundation for classification (van der Velden 2017). In an attempt to overcome these issues, the International Workshop for a Classification of Periodontal Diseases and Conditions introduced a replacement classification system ten years later (Armitage 1999). The 1999 classification system focused on the periodontitis progression rate. A case on periodontitis was classified as chronic if the alveolar bone loss had progressed slowly, and as aggressive if bone loss had progressed rapidly.

Although this system was utilised across the world for the next 18 years, clinicians and researchers still experienced problems with its application. As the classification system had not really introduced any conceptual changes, it was still difficult to differentiate between chronic and aggressive periodontitis. This meant that data produced by different research centres could not be combined. The 2017 World Workshop proposed a new classification system for periodontal diseases. The European Federation of Periodontology (EFP) and the American Academy of Periodontology (AAP) participated in this workshop with the aim of developing a simpler system for general practitioners and periodontists. This system still did not differentiate between chronic and aggressive periodontitis as there was lack of evidence concerning their differences. However, the system categorised classical localised juvenile (aggressive) periodontitis as an individual phenotype. The classification system, which was introduced in 2017, specified the clinical attachment loss (CAL) as a factor for characterising the type on periodontitis, periodontitis, necrotising periodontitis or a manifestation of systemic disease. The system also specified the progression rate of periodontal disease, which are stage I – IV (severity and extent of periodontal tissue destruction) and grade A – C (future risk of progression) (Papapanou et al. 2018, Tonetti et al. 2018).

The 2017 system also included grading parameters that could be used to evaluate the probability of periodontitis progression. However, the reliability of the parameters was not high enough to indicate when the active phase of periodontitis would occur, even when diabetes- and smoking-related risk factors are considered (Leite et al. 2018, Nascimento et al. 2018). Therefore, Tonetti et al. (2018) pointed out that there was still a need for more robust biomarkers to be introduced, which would allow clinicians to identify whether or not a case of periodontitis is in its active phase (continuing periodontal breakdown). This would also allow clinicians to monitor the progress of the disease and make decisions concerning appropriate treatment methods.

There has been extensive research into the possibility of using oral fluids, such as GCF, saliva and mouth rinse, to find biomarkers that could be used to diagnose periodontitis. In recent decades, there have been several researchers searching for suitable biomarkers that can detect, predict and reflect the pathophysiology of periodontal and peri-implant diseases. However, a biomarker that has received a lot of research attention is MMP-8. A number of studies (Lee et al. 1995, Romanelli et al. 1999, Kiili et al. 2002, Sorsa et al. 2006, Al-Majid et al. 2018, Sorsa et al. 2020) have reported that elevated levels of active MMP-8 are a precursor of periodontal attachment loss and can provide insight into making a distinction between gingivitis and periodontitis. The same is not true for latent or total MMP-8.

The use of point-of-care (PoC)/chair-side tests, which are based on aMMP-8 measurements, is demanding. Recently, simple, yet sophisticated, tools (PerioSafe/ImplantSafe with the quantitating Oralyzer reader) have been introduced and validated by different research groups. One of the advantages of this method is that samples of oral fluid can be collected non-invasively, and unusually high levels of aMMP-8 indicate that the active phase of periodontal disease is in progress.

The previously mentioned tool has been used successfully to identify active periodontal tissue destruction, periodontitis, peri-implantitis and subclinical periodontitis – all forms of active periodontal disease (Nwhator et al. 2014, Al-Majid et al. 2018, Sorsa et al. 2016, Zhang et al. 2018, Raivisto et al. 2019, Sorsa et al. 2020). These terms are discussed in more detail in the methods and discussion section.

2.4 PERIODONTITIS

2.4.1 Structure of healthy periodontium

The periodontium is the tissue supporting the teeth; it consists of soft and hard tissues: 1) the gingiva, 2) the periodontal ligament, 3) the root cementum, and 4) the alveolar bone. Gingival tissues, which form a ring of masticatory mucosa around the teeth, protect against infection and friction caused by mastication. Healthy periodontal tissues are degraded

and repaired in a balanced manner so that the functional and structural integrity of the periodontium is preserved. The gingiva is composed of a densely collagenous tissue, the lamina propria, which contains blood and lymphatic vessels and nerves, supra alveolar fibres, and three types of epithelia. (1) The oral epithelium faces towards the oral cavity (2) the sulcular epithelium faces towards the teeth, and (3) the junctional epithelium (JE) is located in the area from the cementoenamel junction to the bottom of gingival sulcus; it is a thin and non-keratinised tissue with wide intercellular spaces through which inflammatory cells can move (Nanci and Bosshardt 2006, Carranza et al. 2011). The cells of the JE's internal basal lamina form the epithelial attachment to the tooth surface with junctional complexes called hemidesmosomes. The cellular turnover is high within the JE because the tissues are constantly remodelled (Nanci and Bosshardt 2006, Carranza et al. 2011). These allow neutrophils to transmigrate through the JE and reach the gingival crevice, creating a defensive barrier against microbes (Tonetti et al. 1998, Darveau 2010). Even if the periodontium is healthy, around 30,000 neutrophils pass through the periodontal tissue every minute (Schiott and Loe 1970, Tonetti et al. 1998). People who have neutrophil deficiencies, whether acquired or congenital, are very likely to develop periodontitis (Hart et al. 1994).

The periodontal ligament, although proportionally small, has an important function. It forms a junction between two hard tissues; the teeth and the alveolar bone (McCulloch et. al 2000). Collagen type I is the main type of collagen in the periodontal ligament, and it is also considered to be the main substrate of MMP-8. Chewing forces are absorbed by the periodontal ligament, and nerve receptors within the ligament send feedback about the forces to the brain (McCulloch et al. 2000, Nanci and Bosshardt 2006). The periodontal ligament also supplies nutrients to the periodontium and plays a part in the remodelling of tissue, alveolar bone, and cementum. (Embery 1990, Nanci and Bosshardt 2006).

Cementum has the principal function of connecting the teeth to their surrounding alveolar bone. Cementum is not remodelled but a slow apposition continuously occurs in accordance with physiologic demands. Alveolar bone is a relatively unstable structure; a characteristic that may contribute to the pathogenesis of periodontitis. It has a high turnover rate, but when in a healthy state there is a balance between the resorption and formation of new bone. (Nanci and Bosshardt 2006). See figure (2) in Section 2.5 (Implantitis).

2.4.2 Role of aetiologies and pathogenesis in periodontal diseases

There is a multifactorial aetiology behind periodontitis, making it a complicated disease. Starting with gingival inflammation (gingivitis), which is mainly caused by bacteria adhering to the tooth surface and forming colonies. It can be identified by redness, swelling and bleeding. Gingivitis is reversible with appropriate treatment. If gingivitis is left untreated, it can result in more severe inflammation, and the alveolar bone, periodontal ligament and tissues can begin to degrade and become irreversible. The key marker in the disease of gingivitis, when the disease is likely to transform to periodontitis, is when JE is converted

to epithelium pockets (Socransky et al. 1998). Periodontal pockets are the clinical sign of periodontitis. A periodontal pocket refers to abnormal sulcus depth between the tooth and periodontal tissues. It is formed when the sulcular epithelium migrates abnormally across the surface of the teeth. This pocket is conducive to the accumulation of biofilm and the growth of anaerobic bacteria. When gram-negative microbial plaques colonise the deepened gingival crevice, a chronic inflammatory response is initiated. In periodontitis, the subgingival biofilm, which in its healthy state is a symbiotic community dominated by gram-positive bacteria, is transformed to a dysbiotic state dominated by gram-negative bacteria. Particular species of bacteria are often observed together in subgingival plaque (Socransky et al. 1998). Among these species, there are some that are strongly associated with periodontitis, particularly the "red complex", which includes *P. gingivalis*, *T. denticola* and *Tannerella forsythia* (*T. forsythia*). Although these bacteria are not necessarily the cause of the disease, they cause inflammation and periodontal pockets, which are favourable conditions for periodontitis-associated pathogens (Wade 2013, Hajishengallis et al. 2014, 2015).

Currently, scholars have found that there is a variety of microbiota found in periodontitis, and red complex organisms are also present when there is no disease (Kononen et al. 2007, Wade 2013). The existence of particular pathogens or a large amount of plaque do not seem to explain the severity of periodontitis experienced by individuals, but their response and susceptibility to the pathogens play a significant role. For instance, if one person is particularly genetically susceptible to the disease, tissue destruction can result from just a small amount of biofilm. Recent research has defined periodontitis as a polymicrobial disturbance of host homeostasis (Darveau 2010), and pathogenesis has been described with a poly microbial synergy and dysbiosis model (Hajishengallis and Lamont 2012).

These studies have reported that periodontitis is described by a synergistic and dysbiotic polymicrobial community, not simply the existence of bacteria, which have generally been termed as periodontal pathogens. Keystone pathogen hypothesis suggests that a number of pathogens, such as *P. gingivalis*, may be responsible for symbiotic microbial communities in the mouth being transformed into dysbiotic populations (Hajishengallis et al. 2012, 2015, Bosshardt and Lang 2005, Noh et al. 2013).

When dysbiotic microbes accumulate in the subgingival area, there is a corresponding proliferation of key molecular patterns (pathogen-associated molecular patterns, or PAMPs) such as lipopolysaccharides (LPS), lipoteichoic acids, peptidoglycan and glucans, which the immune system recognises as threats (Hernandez et al. 2011, Yucel-Lindberg and Bage, 2013). Host cells that have been attacked or injured by trauma or the invasion of bacteria may secrete endogenous molecules known as danger-associated molecular patterns (DAMPs) to alert the immune system to the presence of danger (Jun et al. 2017). These signals are recognised by pattern recognition receptors (PRRs), the most important of which are toll-like receptors (TLRs). Patients with periodontitis show a higher expression

of TLR 2, -3, -4, and -9 in gingival tissue than healthy individuals (Rojo-Botello et al. 2012). PAMP/DAMP-PRR interaction results in the production of pro-inflammatory cytokines and the instigation of inflammatory reactions (Pollanen et al. 2012, Bosshardt et al. 2018). The presence of bacteria leads to the recruitment of neutrophils, which are the body's first line of defence against bacterial proliferation (Wellappuli et al. 2018). The cells that form part of the immune system also secrete complement proteins (Olsen et al. 2017). These proteins contribute significantly to immune and inflammatory responses through adaptive and innate immunity. Some of them are also able to recruit neutrophils. While promoting inflammation, some periodontal bacteria may not be killed by the neutrophils and may even cause the death of neutrophil cells, and thus, contribute to dysbiosis (Olsen and Hajishengallis 2016).

If neutrophils, as the first line of defence against microbe invasion, fail in their function, greater numbers of chronic inflammatory cells such as macrophages and lymphocytes are recruited, thus extending the period of inflammation (Cortés-Vieyra et al. 2016). This is followed by several pro-inflammatory cytokines being released, including IL-6, IL- 8, and TNF-α (Noh et al. 2013). IL-8 is a strong chemoattractant cytokine, and when inflammation, occurs its function is to activate neutrophils. A number of different endothelial cells can release IL-8, including phagocytes, gingival fibroblasts, monocytes and neutrophils (Finoti et al. 2017). IL-1β is a pro-inflammatory cytokine and it has an important role in the regulation of the inflammatory response (Dinarello 2011). The expression of these cytokines intensifies inflammation leading to even more tissue being destroyed (Pollanen et al. 2012, Bosshardt et al. 2018). IL-1β has a number of functions. It increases the expression of intercellular adhesion molecule 1 (ICAM-1) in endothelial cells and mediates the secretion of chemokine (IL-8). Therefore, it facilitates neutrophils' infiltration into the site of inflammation. Bone resorption is induced by IL-1β, along with other proinflammatory cytokines and prostagaldin E2 (PGE2). Significantly elevated levels of IL-1β have been detected in periodontitis and peri-implantitis (Alexander and Damoulis 1994, Ghassib et al. 2019). IL-1β can induce MMP expression and production, particularly MMP-8 (Sasaki et al. 2000, Dinarello et al. 2011, Silva et al. 2015). TNF-α and IL-1β promote the secretion of IL-6, which is produced by a number of immune cells, such as dendritic cells, T-cells, macrophages and B-cells, in addition to resident cells such as fibroblasts, endothelial cells and keratinocytes. Osteoclasts also secrete IL-6, which stimulates bone resorption and osteoclast development. Patients with periodontitis show high levels of IL-6 in their GCF, tissues and cells (Guillot et al. 1995, Ghassib et al. 2019).

2.4.3 Host response

When periodontitis occurs, it results in dysregulation of the host immune response and disruption of tissue homeostasis. The first of these may originate from the challenge caused by the previously mentioned keystone pathogens and pathogenic microbial state, or immunoregulatory defects that can be caused by lifestyle habits, such as smoking, or condi-

tions, such as diabetes. When dysbiotic microbiota are detected, as a defence against these microbes, the periodontal tissues express IL-8, intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule (E-selectin). These allow neutrophils to transmigrate through the JE and reach the gingival crevice, creating a defence barrier against the microbes (Tonetti et al. 1998, Darveau 2010, Loos et al. 2020). The increased production of cytokines, chemokines and proteolytic enzymes results in increased inflammation. Periodontal tissue destruction is mediated mainly by neutrophils. Patients with periodontitis have been observed to have a large accumulation of neutrophils (Pollanen et al. 2012), but if there are too many neutrophils, this can cause periodontal tissue destruction through the release of degradative enzymes, such as MMPs, expressly MMP-8 and MMP-9 (Herr et al. 2007, Sorsa et al. 2016, Morais et al. 2018), elastase, cathepsins, and cytotoxic substances, such as ROS.

This in turn results in inflammation-mediated bone loss; RANKL is expressed and this induces osteoclastic bone resorption (Cortés Vieyra et al. 2016). In healthy tissue, MMPs and their inhibitors TIMPs, such as TIMP-1, play a significant role in the ECM homeostasis. An imbalance between MMPs and TIMPs could lead to the destruction of tissue in inflammatory diseases, such as periodontitis and peri-implantitis where there is an increase in MMP expression and secretion, and a reduction in the levels of TIMPs (Offenbacher et al. 2007, Yucel-Lindberg and Bage 2013). As periodontitis progresses in the inflammation site, lymphocytes, macrophages and antigen-presenting cells (APC) also become active and transmigrate to the site of inflammation. The inflammation increases because inflammatory cells and gingival epithelial cells express IL-1β, IL-6, and TNF-α. This results in the host cells secreting MMPs, which leads to the destruction of Booth soft and hard periodontal tissues. Therefore, it can be concluded that host-derived factors bear primary responsibility for the destruction of tissue in periodontitis (Darveau 2010, Hajishengallis et al. 2012, 2015). See figure (1).

2.4.4 Periodontitis risk factors and evidence for association to systemic diseases

A number of risk factors have been established for periodontitis, including genetic factors, gender (with males being more susceptible), poor oral hygiene, elderly age, and smoking (Mantyla et al. 2003, Genco and Borgnakke 2013). It has also been suggested that systemic and other behavioural factors may play a role in this condition, for example, unhealthily diet, excessive alcohol consumption and stress. Certain medical conditions can contribute to the risk of periodontal disease, such as osteoporosis, obesity and diabetes. Many of these factors, e.g., stress, diabetes and smoking, do not act as initiators of the disease, but they alter the immune response of the host (Knight et al. 2016). The most common genetic factor linked to increased periodontitis susceptibility is polymorphisms in immune response genes (Loos et al. 2005). It is thought that genetics play a more significant role in aggressive and early-onset periodontitis compared to chronic periodontitis. The inter-

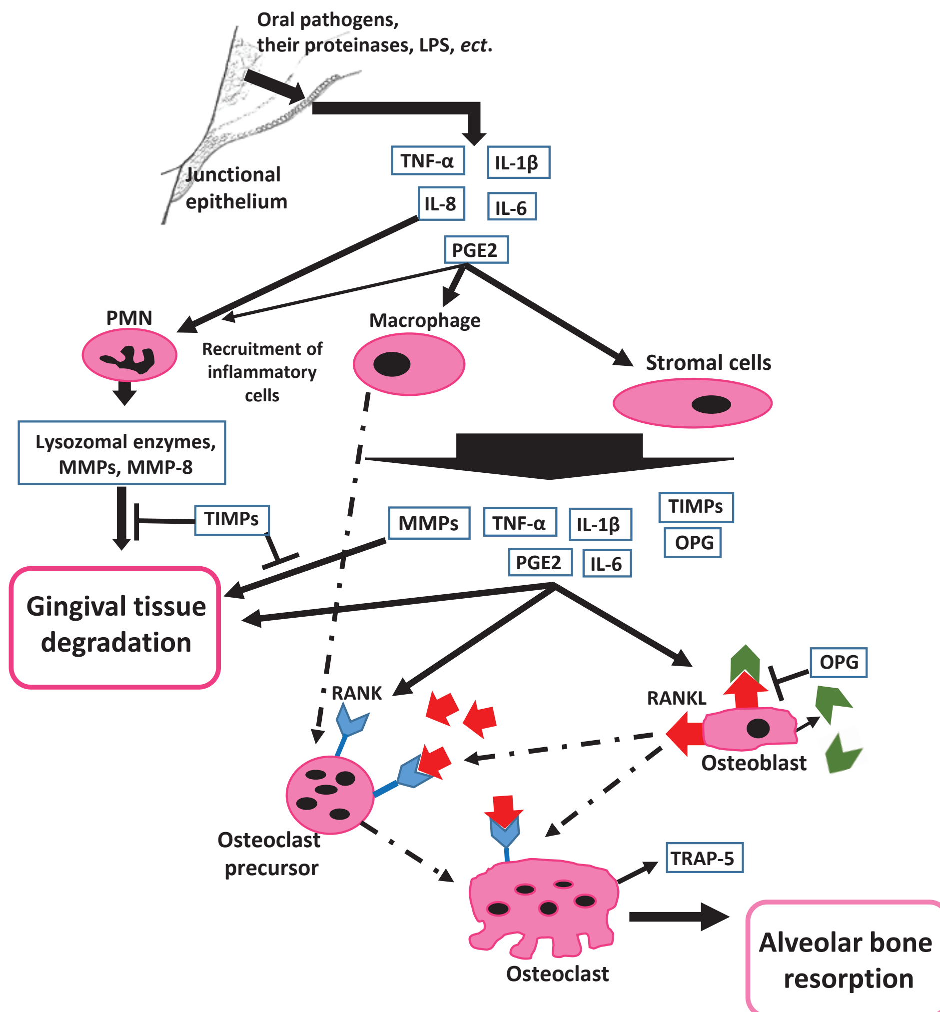

Figure 1. The host response in periodontitis is a cascade process between cell types and inflammatory mediators, some of them are pointed in this figure. Notable are, cytokines and PGE2 affect the expression of RANKL, when the level of RANKL is increased, the OPG is not any more able to inhibit the interaction between RANKL and RANK, resulting in formation, activation of osteoclasts and alveolar bone degradation. TRAP-5 is secreted from osteoclasts as a consequences of bone resorption. IL, interleukin; PGE2, prostaglandin E2; PMN, polymorphonuclear leukocyte; MMP, matrix metalloproteinase; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; TRAP, tartrate-resistant acid phosphatase. Figure modified after (Yucel-Lindberg and Bage, 2013).

actions between all of the risk factors contribute to complex inter-relationships between them. One example is excessive alcohol consumption – this is linked to a low socioeconomic status and poor oral hygiene and, therefore, it affects the immune response and the individual's systemic health. As a result of this, it can be very challenging, if not impossible, to establish single risk factor-based causality (Heaton and Dietrich 2012). On a more serious note, periodontitis can have a negative impact on systemic health. Multiple studies have confirmed associations with conditions such as diabetes, cardiovascular diseases (CVD) and adverse pregnancy outcomes (Linden et al. 2013, Reyes et al. 2013, Kornman 2013, Morais et al. 2018, Chaparro et al. 2020).

Cytokines, such as TNF-α, IL-1β and IL-6, can be released by host inflammatory cells that have been stimulated by an inflammatory agent. This can result in the liver stimulation and the secretion of acute phase reactants; wherein C-reactive protein (CRP) is a primary reactant. It was found that patients with periodontal disease had elevated plasma CRP levels following meta-analysis re-evaluation in a cross-sectional study. The researchers Paraskevas et al. (2008) agreed on this finding when comparing subjects with clinically healthy periodontium. According to Vidal et al. (2009), it was made evident for the first time, following a recent randomised clinical trial, that non-surgical periodontal treatment led to decreased plasma CRP and IL-6 levels in patients suffering from severe periodontitis and severe non-responsive arterial hypertension.

Heightened circulating MMP-8 levels have been linked to obesity, which emphasises the understanding that obesity may be a subclinical inflammatory condition (Lauhio et al. 2016). Smoking also raises MMP-8 levels (Mantyla et al. 2006). Lauhio et al. (2016) have indicated that high MMP-8 levels in obese people may be a contributory factor in the advancement of insulin resistance, wherein cleavage of insulin receptors occurs. A synthetic MMP-8 inhibitor can restrict this. Such results indicated MMP-8 to be a vital mediator in the systemic inflammatory response linked to obesity, and therefore a potential drug target. The deployment of MMP inhibitors may reduce the long-term mortality that is linked to such subclinical inflammatory conditions. The cleavage of the human insulin receptor (INSR), which is instigated by MMP-8, can be restricted with synthetic inhibitors, such as doxycycline.

According to studies carried out by Cervino et al. (2019), Fiorillo et al. (2019), elevated aMMP-8 levels were observed in diabetic patients (type 1 and 2), and both diabetes and pre-diabetes are very much linked to periodontal disease, gingivitis and periodontitis. Seppala et al. (2006) have investigated how collagenase can be activated in diabetic patients' GCF samples. Periodontal tissue destruction is strongly linked to insufficiently regulated diabetes, and when there are collagenases present in GCF, this effect can be both reflected and mediated. The study researchers have suggested that, if levels of blood glucose are diagnosed early and strictly regulated, this will contribute to controlling diabetic patients from contracting periodontal diseases. The study also indicated that periodontal tissue destruc-

tion in people suffering from type 1 diabetes is likely to be mediated at least in part by active collagenases.

Recent research by Grigoriadis et al. (2019) confirmed that, increased aMMP-8 levels are associated more often with pre-diabetic patients. The patients have been diagnosed by means of clinical protocol with chairside workflow to identify undiagnosed hyperglycaemia in periodontal clinics. The PerioSafe test produced contributory evidence that the clinical dental environment offers a good opportunity to screen for diabetes. An aMMP-8 test is a convenient and efficient method for identifying previously undiagnosed diabetes.

Some other biomarkers have received attention from researchers because of their significant association with periodontitis and other systemic diseases. One of these biomarkers is asymmetric dimethylarginine (ADMA), which is commonly referred to in cardiovascular and hypertension research. In terms of periodontitis, this biomarker can be measured in both oral fluids and blood/plasma (Tsioufis et al. 2010, Isola et al. 2020). Macrophage inflammatory protein 1α (MIP-1α) is another inflammatory biomarker, which has been studied in periodontitis in connection with systemic diseases (Al-Sabbagh et al. 2012). Oxidative stress is defined as an increase in oxidant production or insufficient antioxidant production. An imbalance of oxidants/antioxidants leads to excessive ROS generation, which can cause various forms of periodontal tissue destruction (Gumus et al. 2015).

Vitamin C is an important nutrient, and it is acritical dietary oxidant in periodontal health. It acts as an anti-oxidant and reducing agent, which scavenges ROS, including free radicals. Vitamin C induces the differentiation of periodontal ligament progenitor cells by slowing and preventing the progression of periodontal disease. It has been recognised to prevent the oxidative activation of proMMP-8 through its connection to the oxidative MMP activation cascade (Suomalainen et al. 1991, Tomofuji et al. 2009, Tada and Miura 2019).

2.5 PERI-IMPLANTITIS

2.5.1 Definition of implantology

For the last 30 years, edentulous and partially edentulous patients have been offered alternative forms of implantology as treatment. In the last ten years, there has been an increase in the use of implants as abutments for fixed or removable prostheses. The reason for this is that they have superior function and aesthetics and can offer outstanding long-term prognosis and durability. However, dental implants can still fail. If bone loss has occurred, the long-term prognosis of dental implant reconstructions are significantly reduced (Meffert et al. 1996, Xu et al. 2008). Figure (2).

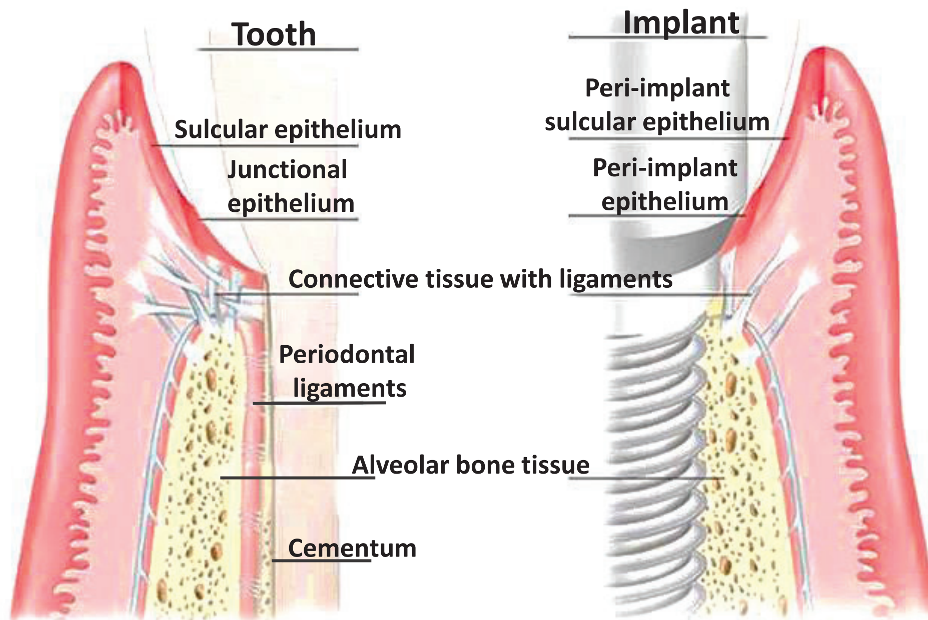

Figure 2. Differences between tooth and implant structures. Figure modified (Carrenza's clinical periodontology, 12th ED chapter 71, figure 71-16. Firolleni et al).

2.5.2 Definitions of peri-implant mucositis and peri-implantitis

Peri-implant mucositis is defined as an inflammatory lesion of the mucosa adjacent to an endosseous implant with no associated bone loss. The two salient characteristics of peri-implant mucositis include the lack of peri-implant bone loss and inflammation in the peri-implant mucosa. Inflammation can be seen during a clinical examination because the tissue bleeds on probing, and there may also be signs of suppuration, swelling and erythema (Lindhe et al. 2008, Heitz-Mayfield et al. 2010, 2018).

On the other hand, while peri-implantitis also affects the tissue that surrounds dental implants, its main characteristics are inflammation in the peri-implant mucosa and bone loss. The inflammation can be detected as described above, however, radiographs are required to determine whether or not bone loss has occurred. In the same way that gingivitis leads to periodontitis, it is assumed that peri-implant mucositis occurs before peri-implantitis (Mombelli and Lang 1998, Lang et al. 2011, Schwarz et al. 2018).

2.5.3 Pathogenesis of peri-implantitis

Although peri-implantitis and periodontitis possess similar etiological factors and pathogenesis, there is an important difference between the structure of the tissues surrounding an implant and that of the periodontium around natural teeth (Heitz-Mayfield et al. 2010).

Peri-implantitis and chronic periodontitis are associated with similar bacterial flora. Studies have found that there are high concentrations of gram-negative anaerobic bacteria and periodontitis-related pathogens in the vicinity of implants that show signs of peri-implantitis (Sumida et al. 2002, Quirynen et al. 2006, Agerbaek et al. 2006).

The similarities in the aetiology and pathogenesis of periodontitis and peri-implantitis imply that there are more similarities than differences between the two diseases (Heitz-Mayfield et al. 2010). Both diseases are initiated by contact with a biofilm that holds pathogens, and both are associated with microbiota that have high gram-negative bacteria levels (Botero et al. 2005, Shibli et al. 2008, Tabanella et al. 2009). However, there is evidence to suggest that some instances of peri-implantitis involve the pathogen *Staphylococcus aureus* (*S. aureus*). Further research is required into the role played by *S. aureus* and other putative pathogens in peri-implantitis progression (Kronstrom et al. 2001, Botero et al. 2005). Although there are strong similarities between the response of the host to the bacterial challenge in gingivitis and peri-implant mucositis, the inflammatory response to persistent biofilm accumulation is more pronounced in peri-implant mucosal tissues than in dentogingival units. This could be due to differences in their structure including, for example, the ratio of fibroblasts to collagen, or vascularity. The "epithelial sealing" around both natural teeth and implants performs a similar function (Gould et al. 1984, Atsuta et al. 2015). Moreover, the structural differences that exist between teeth and implants have not been found to influence the host response to bacterial challenge (Zitzmann et al. 2001, 2002).

Studies have shown that peri-implantitis and periodontitis lesions have many common factors (Konttinen et al. 2006, Berglundh et al. 2011, Belibasakis et al. 2014). Many inflammatory cells are infiltrated in the connective tissue situated next to the pocket epithelium, in particular plasma cells and B-lymphocytes. In both periodontitis and peri-implantitis, the upregulation of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and the TNF-α occurs (Duarte et al. 2009, Javed et al. 2011). However, one important difference has been reported where the infective lesions progress very rapidly and reach the alveolar bone marrow in certain stages of peri-implantitis. A recent study compared the two diseases and concluded that natural teeth had a "self-limiting" process in the surrounding tissues. During this process, "a protective connective tissue capsule of the tooth's supracrestal gingival fibres separating the lesion from the alveolar bone" is created. This did not occur in peri-implant tissues, where the lesion was able to spread to the bony crest (Zitzmann et al. 2004, Berglundh et al. 2011). Therefore, we can make the reasonable assumption that in humans with peri-implantitis, there are destruction periods that are far more rapid than those seen in chronic periodontitis (Berglundh et al. 2011).

2.6 TREATMENT OF PERIODONTITIS AND PERI-IMPLANTITIS

In general, both periodontal and peri-implant treatment aims to re-establish the homeostatic state, and thereby rebuild the reciprocally beneficial relationship between the community of microbes in the oral cavity and periodontal tissue (Khan et al. 2015). Treatment for periodontitis and peri-implantitis is based on removing the plaque retention and reducing the biofilm. Ultrasonic and manual instruments are utilised to debride the subgingival biofilm and granulated tissues using SRP (Sanz et al. 2012, Belibasakis et al. 2014). Chlorhexidine, a local antimicrobial agent, may also be administered. A new natural fermented lingonberry juice mouthwash has shown promising results in balancing microbial flora and decreasing the signs of inflammation (Parnanen et al. 2019). However, if bone loss has occurred and peri-implantitis has developed, non-surgical treatments are not effective. If the case is particularly severe, it may be necessary to prescribe surgical therapy or systemic antibiotics, although with caution in order to prevent antibiotic resistance (Herrera et al. 2012). For periodontitis to be successfully treated and prevented from reoccurring, patients must be given instructions on how to maintain oral hygiene and, in the case of smokers, smoking cessation counselling should be offered. As a further preventative measure, and to maintain the positive outcome of treatment, patients must have regular appointments for receiving adjunctive periodontal therapy as the annual report of American Academy of Periodontology 2000 suggest.

2.6.1 Low-dose-doxycycline/sub-antimicrobial-dose-doxycycline (LDD/SDD)

Increasing resistance to antibiotics encouraged researchers to find alternative procedures and different treatment strategies to avoid serious consequences (Isola et al. 2018). Medications, such as tetracyclines, have also been found to inhibit MMPs (Golub et al. 2001, Emingil et al. 2004). Low-dose-doxycycline/sub-antimicrobial-dose-doxycycline (L/SDD) therapy aims to re-establish a balance between pro-inflammatory and anti-inflammatory mediators. Host modulation with L/SDD medication decreases gingival tissue and aMMP-8 levels in oral fluid. It can also halt periodontal/peri-implant tissue destruction (Golub et al. 2016, Emingil et al. 2019). Doxycycline plays an essential role in MMP inhibition by means of the downregulation of gene expression, suppression of catalytic activity and halting oxidative activation (Uitto et al. 1994, Smith et al. 1999). Currently, L/SDD is the only MMP-inhibitory drug for humans that has been licensed by the US Food and Drug and Administration (US FDA) for the treatment of periodontal disease (Golub et al. 2016). The treatment consists of a 20 mg doxycycline dose, which is administered orally twice a day, or alternatively, a 40 mg dose once a day. The treatment produces serum levels of doxycycline that are too low to generate any antimicrobial effects, yet sufficient to inhibit/downregulate aMMP-8 (Sorsa et al. 2016). Compared to the higher traditional dose of 100 mg (once or twice daily), it is considered that L/SDD will not to result in bacterial resistance to doxycycline, and it does not significantly alter normal flora, even following long-term daily intake (up to 24 months) (Golub et al. 2016). Furthermore, L/SDD significantly reduces inflam-

matory mediator levels, aMMP-8, collagen degradation, pro-inflammatory cytokines, connective tissue destruction in the periodontal region, and alveolar bone loss (Preshaw 2004, Preshaw et al. 2008). MMPs are considered to play a key role as mediators in periodontal tissue destruction (Kinane 2000, Sorsa et al. 2004, Kinane et al. 2017, Franco et al. 2017), and the irreversible connective tissue degradation seen in periodontitis has been reported to occur as a result of an imbalance between MMPs and their TIMP inhibitors (Sorsa et al. 2004, 2016).

2.7 BIOMARKERS

A biomarker is a parameter that evaluates and assesses the index of normal biological and pathological signs, and pharmacological therapeutic response (Strimbu et al. 2010). Making a physical examination of a patient's periodontal and peri-implant tissues is time-consuming and has to be carried out by well-trained dental professionals, which also makes it costly. Biomarkers are still required in periodontal disease; proper biomarkers should indicate disease onset, current inflammatory activity levels and predict the future progression of diseases as opposed to radiographs, in which the visible damage could have been caused by a previous incident of periodontitis (Buduneli and Kinane 2011). The use of biomarkers could help to detect cases of periodontitis and peri-implantitis at an early stage and be used to determine how a patient is responding to treatment and which sites still contain active disease progression. On the other hand, the use of radiography and clinical examinations may not provide accurate results in terms of a patient's susceptibility to periodontitis or how they are responding to treatment because some factors, such as microbial composition and host response, cannot be measured by these interventions although they play a significant role in disease progression (Offenbacher et al. 2008).

Examples of biomarkers for the diagnosis of periodontitis and peri-implantitis are MMPs, host-derived markers, such as cytokines; tissue breakdown products, and markers derived from pathogens, such as bacterial DNA and proteases. Biomarkers in oral fluids, such as saliva, mouth rinse, GCF or PISF, can be easily collected. GCF and PISF are fluids derived from serum, which contain locally produced molecules, such as inflammatory mediators and by-products of tissue breakdown, and systemically derived markers (Recker et al. 2015, Barros et al. 2016). When inflammation occurs, the blood, GCF and PISF flows increase in the periodontal and peri-implant tissues, along with vascular permeability. Paper strips are usually used to collect fluid samples from gingival sulcus or periodontal pockets. Biomarkers in GCF or PISF reflect the status of a particular site in the mouth, whereas mouth rinse and saliva samples provide information on the overall oral status.

Comprehensive research has been conducted on biomolecules in oral fluids, to determine which ones could potentially be used as biomarkers in the diagnosis of periodontitis. It is difficult to specify a single marker of disease activity due to the complicated, multifactorial nature of periodontitis (Miller et al. 2006, Buduneli and Kinane 2011, Chojnowska et al.

2018). Biomarkers should meet a number of criteria: a) accuracy, b) reliability, and c) therapeutic impact and usefulness for early intervention (Redberg et al. 2003). Biomarkers have the great advantage of being able to predict disease activity and they can be used to develop personalised medicine. They are valuable for identifying patients at high risk of a disease, and for predicting outcomes. All biomarkers need validation, and there is still debate as to which biomarker can be determined to be sufficient for clinical use (Lin et al. 2009). If they are being used as diagnostic tools, then their sensitivity and specificity must be assessed.

2.8 MMP-8 IN PERIODONTITIS AND PERI-IMPLANTITIS

MMP-8 is an important contributor to the pathogenesis of periodontitis, and it can also be used as a biomarker for peri-implantitis (Sorsa et al. 2006, Al-Majid et al. 2018, Lupi et al. 2019). There is evidence to show that tissue destruction of tissue occurring in periodontitis and peri-implantitis are mainly due to pathologically excessive collagenolytic activity. Collagen is the major structural protein in periodontal tissues so, as expected, MMP-8 is the most neutral proteinase capable of initiating and stimulating the digestion of collagen type I, which is found in abundance in peri-implant and periodontal tissues (Ling et al. 2009, Arakawa et al. 2012, Leppilahti et al. 2014, Schmalz et al. 2019).

MMP-8 can also play an important role as a diagnostic biomarker for peri-implantitis because it is the most predominant collagenolytic proteinase found in inflamed gingiva and GCF (Sorsa et al. 2016). In terms of evaluating inflammation levels and tissue destruction adjacent to implants, PISF may be able to offer a similar function as that of GCF in natural teeth (Alassy et al. 2019). Elevated MMP-8 levels, in particular in the active MMP-8 form, have been found to be linked to peri-implant and periodontal inflammation or disease, especially during the clinically active phase (Lee et al. 1995, Romanelli et al. 1999, Al-Majid et al. 2018). Suggestions have been made in regard to whether interstitial collagenase MMP-8 is the cause of periodontal and peri-implant degeneration, and active periodontal degeneration (APD) rather than bacterial enzyme (Sorsa et al. 1988, Izadi et al. 2015, Heikkinen et al. 2017) The level of active collagenase-2, MMP-8 (but not its latent form) can be used to predict and reflect the progression of peri-implant and periodontal disease (Sorsa et al. 2017, Morias et al. 2018).

A number of studies have shown that in periodontitis patients, oral fluids contain higher aMMP-8 levels of than healthy controls (Lee et al. 1995, Morais et al. 2018). In addition to periodontitis, it has been recognised that in diseased PISF aMMP-8 levels are raised to pathological levels. It has also been reported that neutrophil collagenase or mesenchymal collagenase MMP-8 isoforms (particularly in active phases) exist in the PISF of peri-implantitis patients. (Kivela-Rajamaki et al. 2003, Marcaccini et al. 2010, Mauramo et al. 2018, Alassy et al. 2019).

2.9 IMMUNOASSAY-BASED AMMP-8 DETECTION

The time-resolved immunofluorometric assay, IFMA, with especific sensitive anti-MMP-8 (Medix Biochemica Ltd, Espoo, Finland) established for detecting aMMP-8 has higher diagnostic accuracy than usually commercial MMP-8 ELISA, such as Amersham (GE Healthcare, UK) (Salminen et al. 2014, Leppilahti et al. 2014). Both, the enzyme-linked immunosorbent assay, ELISA, and IFMA are simple to perform, accurate, reproducible, and amenable to automation, however IFMA method appears to be more sensitive. A particular benefit of the IFMA technology is its large dynamic range (Marple et al. 2001). The IFMA selectivity technique recognises activated isoforms of MMP-8, particularly in the active aMMP-8 form (Al-Majid et al. 2018). On the other hand, usually commercial MMP-8 ELISA's detect all MMP-8, including total and latent forms of MMP-8 (Sorsa et al. 2010).

IFMA results were found to correspond with DentoELISA; utilising the same aMMP-8 antibody. But Lepplahti et al.'s study found it incompatible with Amersham ELISA; overall MMP-8 levels in the test were inconsistent with periodontal parameter values (Teronen et al. 1997, Leppilahti et al. 2011, Nwhator et al. 2014). Baeza et al. (2016) study found differentiating periodontitis from healthy sites was less accurate when measuring aMMP-8 levels by means of IFMA. In contrast to other studies, DentoELISA was found to be comparable to IFMA. There was a correlation between aMMP-8 levels when measured by means of IFMA and PD. IFMA and DentoELISA measurements showed an association between CAL and aMMP-8 (Baeza et al. 2016). The same aMMP-8 antibody utilised by DentoELISA and IFMA displays good cross-correlation with lateral-flow chairside/PoC PerioSafe and ImplantSafe immunotests both with and without the quantitative Oralyzer reader (Ramseier et al. 2016, Sorsa et al. 2017, Rathnayake et al. 2017).

2.10 PERIODONTAL CHAIRSIDE TEST KITS

Several kits, which have been designed to discern oral fluid biomarkers using a variety of techniques, identify particular periodontopathogens or carry out an enzymatic reaction of these pathogens, have been created for commercial use commercially or for model-testing. PerioGard, the commercial test kit for measuring aspartate aminotransferase (AST) activity in GCF, aims to give a positive test result concerning GCF AST activity ≥ 800 µl/U wherein the result appears as a colour change after 15 min. The test cannot differentiate well between sites with severe inflammation (Persson et al. 1995, Chepuri et al. 2015).

PocketWatch is a different test, which is used to assess AST levels. The test can detect destructive periodontal pockets and it can also be used for assessing treatment plans. The assessment results can be helpful in clinical reviews of periodontal disease locations (Shimada et al. 2000, Chepuri et al. 2015).

The Periocheck kit is used to detect non-specific neutral proteases and increased proteolytic activity in GCF. The baseline sensitivity for Periocheck was 88% with a specificity of 61%. Therefore, it could not be considered as a reliable reflection in clinical periodontitis measurement (Hemmings et al. 1997).

Perioscan is also a chairside test, which has been devised for detecting a group of periodontopathogens, *P. gingivalis*, *T. denticola* and *T. forsythus* as well as the trypsin-like enzyme produced by pathogens. The sensitivity and specificity of the test were 99% and 55% respectively (Hemmings et al. 1997, Chepuri et al. 2015).

A description of a chairside assay for calprotectin in GSF has also been developed and the test is reliable for diagnosing periodontal diseases. However, it does not have the ability to reflect the the disease severity and activity while there is no substance degradation caused by calprotectin (Kido et al. 2017).

3. AIM OF THE STUDY

The hypothesis of the study was that PerioSafe and ImplantSafe tools with the quantitative Oralyzer reader would be accurate in the detection and prediction of periodontal and peri-implant diseases. We also hypothesised the reliability and efficacy of aMMP-8 PerioSafe test with Oralyzer for grading and staging purposes in the new periodontal classification system. We also assumed the ability of proteome approach (LFQ) for measuring different biomarkers in saliva in order to compare these results with traditional immunoassay-based methods. Finally, we hypothesised the effectiveness of SDD therapy for some inflammatory biomarkers and periodontal clinical parameters.

In the process of achieving these objectives, the study was divided into five more specific goals:

1. The aim of study I was to examine the MMP-8 expression and activation in established gingivitis and to validate LFQ in comparison to antibody-based immunoassays methods.

2. Study II had two main objectives. First, it used PerioSafe and ImplantSafe with the Oralyzer reader to determine how well healthy and diseased patients could be differentiated on the basis of aMMP-8 expression, and whether it could be used to assess the treatment efficacy. Secondly, IFMA and zymography methods were used in PISF samples to evaluate how well healthy and diseased patients could be differentiated.

3. Study III evaluated the aMMP-8 PoC test, PerioSafe with the Oralyzer reader, in the new periodontal classification system (stage/grade) that was published in 2018 in order to compare the effectiveness of the PerioSafe system with traditional BOP and PI methods. It also compared mouth rinse and saliva to determine which of the two was the most sensitive for detecting aMMP-8.

4. Study IV aimed to assess the utilisation of GCF and saliva in the PoC aMMP-8 test for the purpose of diagnosing and differentiating healthy sites, gingivitis and periodontitis in line with the new global classification of periodontitis. The IFMA method was used quantitatively in GCF and saliva samples to show the sensitivity of aMMP-8 detection in both samples.

5. Study V aimed to assess how effective SDD is when combined with non-surgical periodontal therapy. The results were compared to results of non-surgical periodontal therapy when not combined with SDD. It investigated the effects of these therapies over a period of twelve months. The study focused on GCF biomarkers, such as MPO, MMP-9, MMP-13, OPG, and TRAP-5 by ELISA, and MMP-8 by IFMA, for periodontal tissue catabolism that are linked to clinical outcomes.

4. STUDY SUBJECTS AND METHODS

4.1 DESIGNS, POPULATIONS, CLINICAL EXAMINATION, INCLUSION AND EXCLUSION CRITERIA (I, II, III, IV, V)

Study I

This is an experimental gingivitis model; the participants for this study (n=10) were selected from a larger study on human gingivitis, which was carried out by the Department of Dentistry and Oral Health, Aarhus University 2015–2016 (Nascimento et al. 2019). There were three phases in the model. The first phase (14 days prior to the start date of second phase (day 0)) included recruitment and oral hygiene practices. From day 0 to day 21, during the second phase, dental plaque was induced by omitting any oral hygiene-related activities. In the third and final resolution (R) phase, from day 21 to day 35, oral hygiene practices were resumed to remove the plaque.

The participants were all 18-35-year-olds and the inclusion criteria for the study included ≥ 20 teeth, PD ≤4 mm and interproximal CAL <2 mm. Levels of plaque and gingival inflammation were measured and recorded according to the modified Quigley and Hein plaque index (TQHPI) and the modified gingival index (MGI) respectively. TQHPI is an index that measures the plaque disclosed on the unrestored teeth's buccal and lingual surfaces at a scale of 0 to 5, as described by (Quigley et al. 1962) and updated by (Turesky et al. 1970). An index is specified for the entire mouth, except for the third molars, by dividing the total score by the number of examined surfaces. MGI was developed by Lobene et al. (1986). An index was used for visually measuring the incidence and severity of gingivitis without any invasive measures.

Exclusion criteria included pregnancy, breastfeeding, having taken antibiotics or anti-inflammatory drugs in the past 6 weeks before the study began, smoking and diseases that affected the immune system. No stents were applied. In this longitudinal study, we analysed unstimulated whole saliva samples for the total protein concentration using a Qubit protein assay to identify four biomarkers, including MMP-8, TIMP-1, MPO and PMN elastase by means of LFQ, time-resolved IFMA (MMP-8) or different ELISAs the others. Additionally, molecular forms of salivaryMMP-8 were assessed with Western blots.

Study II

This was a case-control study, which was divided into two parts involving three groups: periodontitis and peri-implantitis with healthy control participants. The periodontal status for both participants with periodontitis and healthy controls was assessed by means of *e.g.* CAL, PD, BOP, PI and radiographic X-ray examinations. The inclusion criteria for the first part of the study included at least five sites with PD ≥4 mm, no history of systemic disorders, no history of antibiotics or anti-inflammatory drugs in the past six months and no

history of periodontitis treatment in the past six months. The three groups consisted of 10 chronic periodontitis patients, 30 peri-implantitis patients, and 10 and 30 healthy control subjects corresponding to the two previously mentioned groups. The group of patients with chronic periodontitis, aged 34 – 73 years), consisted of 8 men and 2 women. Healthy controls were periodontally and systemically healthy dental students (22–24-year-olds). The 10 periodontitis patients and the 10-dental student (control subjects) were Finnish, while the 30 peri-implantitis patients and 30 peri-implant healthy control subjects were Swedish. After baseline examination, mouth rinse samples were examined using PerioSafe to detect aMMP-8. The visual PerioSafe test was initially used, followed by the quantitation of aMMP-8 lateral-flow test-sticks with the Oralyzer reader. After that, periodontal treatment scaling and root planning was done in conjunction with anti-infective therapy. Six weeks after treatment, the patients underwent clinical follow-up checks and new PerioSafe tests were completed to determine the effectiveness of the aMMP-8 PerioSafe test before and after treatment.

In the second part of this case-control study, clinical examinations included CAL, PD, BOP, PI, and X-rays were used to diagnose healthy and peri-implantitis sites. The inclusion criteria included at least PD ≥4 mm, BOP, detected alveolar bone loss around the implant in the peri-implantitis site, no history of antibiotics or anti-inflammatory drugs in the past six months. The peri-implantitis patients included 14 men and 16 women in the age group of 39-79-year-olds, while the healthy participants included 23 women and 7 men. After baseline examination, the ImplantSafe aMMP-8 PoC/chairside PISF test was used to identify peri-implantitis sites (n = 29) so that they could be differentiated from clinically healthy peri-implant sites (n = 32). One peri-implantitis sample was missing, and two healthy controls were added for certainty. The peri-implantitis sites were treated surgically. A follow-up was carried out 3 – 6 months after treatment was administered and a new ImplantSafe aMMP-8 test was completed. The quantitative analysis of aMMP-8 in PISF samples was also carried out by means of IFMA, and MMP-9 gelatinolytic activity was analysed by quantitative gelatin zymography.

Study III

This study was performed in collaboration with Aristotle University, Assoc. Prof Dimitra Sakellari. One hundred and fifty participants, consisting of patients and control subjects, were examined at the Periodontology Aristotles University Clinic, Thessaloniki, Greece. Patients were selected for testing according to the screening questionnaire completed by the Centers for Disease Control and Prevention (CDC) USA (Grigoriadis et al. 2019). Participants were 25-78 years old; 74 were men and 76 women. Their aMMP-8 levels were measured with a PerioSafe test and BOP, CAL and VPI results were assessed using the new classification system (Stage I to III and Grade A to C) (Tonetti et al. 2018). Six surfaces of each tooth were assessed using an automated probe (Florida probe, Florida Probe Corporation, Gainesville, FL, USA). A full clinical examination was initially carried out for each patient

in order to evaluate their oral health and signs of periodontitis. After that, the aMMP-8 mouth rinse test by PerioSafe was completed. Inclusion criteria included not taking certain medications such as anti-inflammatories or antibiotics for three months before the study and having no underlying diseases.
We used PerioSafe with the quantitative Oralyzer reader for all 150 participants to measure the aMMP-8 levels in mouth rinse samples. In the same way, we analysed the aMMP-8 levels in saliva using IFMA to show the efficacy and accuracy of both samples among the 150 Greek patients.

Study IV

This is a cross-sectional study carried out in collaboration with Prof Gulnur Emingil, Ege University, Medical School, Izmir, Turkey. This study engaged 80 participants; 18 of them had Stage III periodontitis; 19 had Stage IV periodontitis; a further 21 had gingivitis, and the remaining 22 had clinically healthy periodontium. Inclusion criteria for participants were the following criteria; they had to be free of systemic diseases, including diabetes, cardiovascular conditions and immune system disorders. They were also barred from participating if they regularly took medication that might impact the periodontal tissues, if they had taken antibiotics in the 10-week period before the study, if they were pregnant or if they smoked. Classification of the subjects matched the most recent periodontitis classification, and the people taking part had to provide written consent.
Four classifications were created in regard to their periodontal condition: (1) A group in good health, in which the subjects had PD <3 mm, no gingival recession as there was no adverse periodontal effect, and CAL <2 mm with BOP <10% of full-mouth score examination; (2) The group suffering from gingivitis had different levels of inflammation, they had CAL < 2 mm, but radiographic bone loss as a result of periodontitis was not detectable. If the patient was suffering from interdental CAL, which could be identified in at least two non-adjacent teeth, they could be considered to have periodontitis; (3) The subjects in the P-Stage III group, whose PD >6 mm combined with vertical bone loss >3 mm, had Class II or III furcation involvement with medium-level ridge abnormality; (4) The final set of subjects – the P-Stage IV group – suffered from impaired function in the form of mastication, secondary occlusal trauma and breakdown of posterior bite abilities. The PD, CAL, papillae-bleeding-index (PBI) (Saxer, & Mühlemann, 1975) and PI data were noted at six locations for each tooth. A manual periodontal probe was used to perform PD and CAL analysis. Chairside/PoC analysis of aMMP-8 levels in GCF and saliva was carried out; the analysis was conducted by means of both a lateral-flow immunotest and IFMA to assess the accuracy of the test among the participants.

Study V

This is a clinical randomised follow-up study, which involved a double-blinded, placebo-controlled adjunctive SDD trial that took place over a period of 12 months. Participants included thirty 31-61-year-old patients with chronic periodontitis (stage III) (Tonetti et

al. 2018) from the Department of Periodontology, Dentistry School, Ege University, İzmir. The participants were recruited between the years 2002-2004. The study was divided into 3 phases: screening, treatment and evaluation. First, the participants were recruited and their eligibility was checked. During the first stage of the study, the patients' periodontal status was assessed. A standard manual probe (Williams probe) was used to take measurements at six sites around each tooth. Full mouth PD, CAL, GI and PI scores were recorded. The dental and medical histories of the participants were taken, and results of X-ray examinations, clinical findings and periodontal history were used for the diagnosis of periodontitis. Information on the treatment selected for patients was kept blinded for the last part of the study. The subjects were then randomised in to two groups: SDD (n = 15) or placebo (n = 15).

The baseline examination was used to collect GCF samples and the same clinician performed non-surgical periodontal therapy over several sessions. A local anaesthetic was used to clear tooth and root surfaces of all deposits. During each session with the clinician, all of the patients were instructed on how to brush their teeth and use interdental floss or brushes. For 3 months, the placebo group took a placebo capsule that contained starch while the SDD group took adjunctive SDD (20 mg per capsule). The capsules for each group were identical in appearance. Both groups were advised to take one capsule in the morning and one in the evening, an hour before eating. They were anonymised by being allocated a code number. In order to encourage compliance with the instructions, the participants were given 28 capsules every 2 weeks.

The participants were evaluated 4 times after the baseline examination. The first time was after 3 months, at the end of the SDD or placebo therapy, and the following evaluations took place at 6, 9 and 12 months. At each of these visits, GCF sampling was completed and clinical parameters were assessed. The participants also received maintenance therapy at each visit, and they were encouraged to continue with their oral hygiene routine.

The inclusion criteria included having at least 14 teeth. Periodontitis is also diagnosed through buccal or oral CAL ≥3 mm with pocketing >3 mm detectable in ≥2 teeth. Exclusion criteria included being pregnant or breastfeeding, or excessive smoking or alcohol consumption. Participants were also excluded if they had used antibiotics, other medicines or treatment for periodontitis within the past four months, or if they were hypersensitive to any form of tetracycline. Participants could also not have a history of any systemic disease. The purpose of the study was explained to the participants and they all provided informed consent. In this longitudinal study, we analysed MPO, MMP-9, MMP-13, OPG, and TRAP-5 in GCF using ELISA, and MMP-8 using IFMA analysis.

4.2 SAMPLES COLLECTION

4.2.1 Gingival crevicular fluid (GCF) (IV, V)

Patients who were recruited for dental examination between 8 am – 10 am were asked to refrain from normal cleaning practices, such as brushing and flossing, as well as rinsing. They were also asked not to drink for two hours before providing a sample (Papagerakis et al. 2019). GCF samples were collected from mesiobuccal sites of single-rooted teeth with probing depths of 6–8 mm using rotating quadrants for each participant. A curette was used to remove supragingival plaque from the interproximal surfaces before sampling took place. An air syringe was used to dry the surfaces and they were separated with cotton rolls. In study IV, the aMMP-8 pool rapid test comprised of a dipstick test in protective foil, GCF collection strips and a tube with an elution buffer. The GCF strip was slid into the gingival crevice to a depth of just one or two mm, so as to not harm the soft tissue. The strip was left in place for 30 seconds, then removed and placed into an elution buffer tube. The specimen of elution fluid was kept for further analysis at a temperature of -80ºC. In study V, a GCF strip (ProFlow, Inc., Amityville, NY, USA) was used to collect GCF. Care was taken when inserting the paper so as to avoid mechanical damage to the teeth. Any strips contaminated with blood were thrown away. In study V, an electronic device (ProFlow) was employed to measure the absorbed GCF volume of each strip. The strips were stored at -80ºC., in sterile polypropylene tubes, until they were required for analysis. A standard curve was used to convert the readings from the Periotron 8000 into actual volumes (µl).

4.2.2 Saliva (I, III, IV)

In study I, 210 unstimulated whole saliva samples were collected as samples. The saliva samples were collected from 42 participants five times (on days 0, +7, +14, +21 and +35). In study III and IV, unstimulated saliva samples were also collected. Participants had to have been fasting for 2 h before the samples were taken between 8 am – 10 am. Participants were told to rinse their mouth with tap water twice and wait five minutes after rinsing, before saliva collection, to prevent any dilution. Samples were stored at -80°C until they were required for analysis (Papagerakis et al. 2019).

Mouth rinse and PISF collection samples have been further defined in the following section concerning PerioSafe and ImplantSafe tests (4.3.2).

4.3 POINT-OF-CARE (POC) AMMP-8-SPECIFIC CHAIRSIDE IMMUNOTESTS (II, III, IV)

4.3.1 aMMP-8 lateral-flow immunotest (dip-stick) (IV)

A lateral-flow sandwich immunoassay is based on the utilisation of monoclonal antibodies conjugated to latex particles. The results from the immunochromatographic chairside dip-stick test (Mantyla et al. 2003) are ready in five minutes. Sorsa et al. (1999) outlined

the test's principle, while the clinical testing protocol was described by Mantyla et al. (2003). The dipstick test was immersed in an elation buffer for five minutes, and the resulting colour change showed one or two lines. The test allowed aMMP-8 concentrations in the GCF specimens to be discerned. The fundamental idea of the test (aMMP-8 dip-stick, PerioSafe and ImplantSafe) was to determine if the sample contains aMMP-8 on the basis of antibodies attached to latex particles. The particles are carried by the liquid flow along the test stick passing over the catching zone containing the other MMP-8 antibody. A blue line will appear indicating a positive result while a negative result is indicated by no line. The correct performance of the test is indicated by the appearance of a second blue line. 20 ng/ml is considered the cut-off mark for the PoC test; this point has been indicated to correspond with the visual aMMP-8 PoC test. Proteins emanating from the GCF travel to the buffer, which then moves down the dip-stick and adheres to the blue, antibody-labelled latex fragments on the stick. If there is enough aMMP-8 present, the saliva reveals a blue line. The test is assessed visually and can be considered as valid if a blue line emerges within five minutes. Two lines (test and control) mean that the dipstick reads a positive result; one line is considered negative. 20 ng/ml is considered the cut-off mark for the PoC test; this point has been indicated to correspond with the visual aMMP-8 PoC test.

4.3.2 PerioSafe, ImplantSafe and Oralyzer (II, III)

Based on the invention by Prof Timo Sorsa, recent lateral-flow PoC PerioSafe and ImplantSafe tests, which originate from Finland and further expanded upon in Germany, have been developed on the basis of earlier technologies, and monoclonal antibodies (Mantyla et al 2003, Sorsa et al. 2017). Medix Biochemica Ltd (Espoo, Finland) and Dentognostics GmbH (Jena, Germany) have developed PerioSafe and ImplantSafe and the Oralyzer reader, which are commercially available (Sorsa et al. 2017).

PerioSafe is a mouth rinse test, which can be administered to patients by following the manufacturer's instructions. The procedure begins with a 30-second prerinse with tap water. There is then a wait of 30 seconds, followed by rinsing for a further 30 seconds with the (aqua purificata) test liquid. The next step involves pouring the mouth rinse into a small collection beaker included in the test kit. A syringe, again part of the test kit, is used to draw of three millilitres of the rinse. A filter is then placed on the syringe, whereupon a maximum of four drops are placed on the test stick of a lateral-flow immunoassay system. The result is visible after five minutes when the test stick provides visual results in the form of a colour change, where one blue line indicates a negative (no-risk) result, and two blue lines show a positive (increased risk) result for periodontitis.

ImplantSafe is used in dental implantology as a rapid lateral-flow chromatography immunotest. It is a modern, *in-vitro* diagnostic dip-stick test based on lateral-flow sandwich immunoassay. Highly specific monoclonal antibodies MoAB 8706 and MoAB 8708 are used in the PerioSafe as antibodies conjugated to latex particles. A sterile PISF collection strip, part of the test kit, is used to collect the sample atraumatically directly from the implant

sulcus. To avoid any bleeding from the marginal gingiva, the tip of the collection sampling strip for absorbing PISF is placed into the gingival sulcus for 30 seconds. It is then placed in a test tube containing a buffer solution. The ImplantSafe dip-stick is immersed in the tube for 15 seconds and then left for five minutes.

The Oralyzer reader can then be used to perform a quantitative analysis of both PerioSafe and ImplantSafe tests. An increased risk of periodontitis or peri-implantitis is evident even if the second line is less pronounced. After five minutes, a PoC chairside diagnosis of the PerioSafe and ImplantSafe test sticks can be quantitated using the digital Oralyzer reader. The reader has two compartments for both different sticks. The test stick is placed in the appropriate compartment of the reader, the cover is closed, and the compartment is pushed into the Oralyzer and the check mark is pressed. The levels of aMMP-8 are measured automatically by the Oralyzer's mechanism in five minutes. The results of the qualitative visually assessed plus/minus test are expressed quantitively in ng/ml aMMP-8 PoC/chairside. As stated, the cut-off value (detection limit) for both tests is (20 ng aMMP-8 per ml). A positive result, indicating normal conditions and a healthy status (<20 ng aMMP-8 per ml) is depicted by a single line, whereas two lines indicate an increased risk of periodontitis or peri-implantitis (≥20 ng aMMP-8 per ml). See figures (3 and 4).

Figure 3. The PerioSafe kit contains an aMMP-8 immunotest including a filtered syringe, test liquid (aqua purificata), lateral-flow immunoassay sticks, and a test liquid/mouth rinse collection cup. (A) two lines indicate a positive result, which means the patient is at high risk of periodontal tissue destruction with ≥20 ng/ml. (B) one line indicates a negative result, which means the patient is not at risk of periodontal tissue destruction with <20 ng/ml.

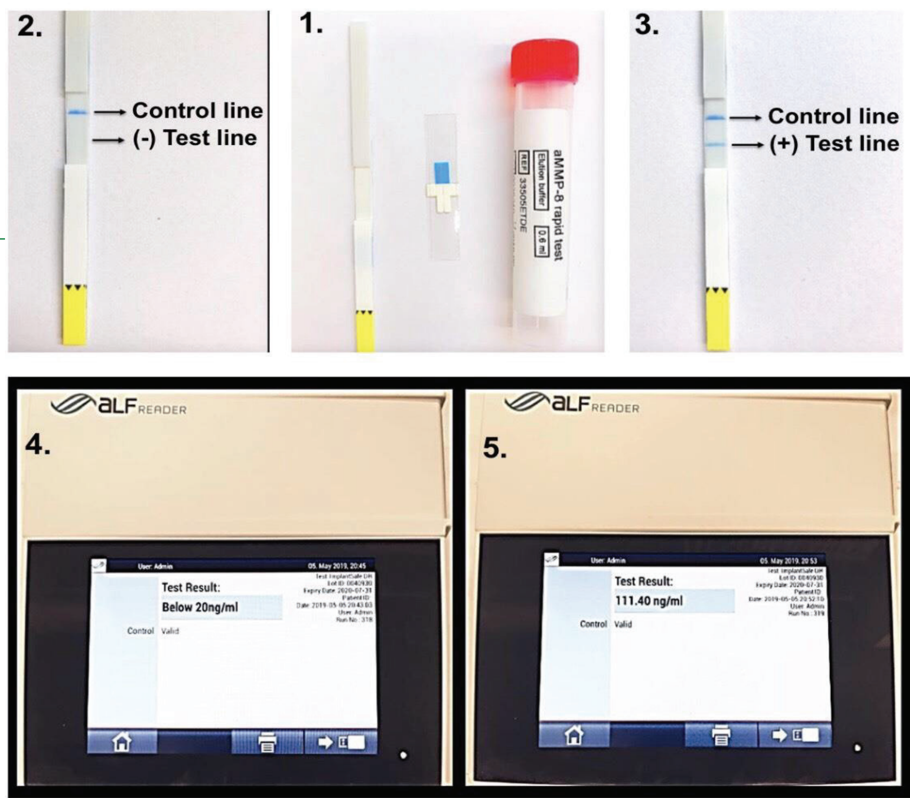

Figure 4. 1. The ImplantSafe kit contains a dip-stick test, PISF collection strip and 600 µl elution buffer. **(2)** results are ready in 5 mins and the appearance of a single line indicates low aMMP-8 in PISF, and no risk of peri-implantitis. **(3)** The appearance of two lines in the result indicate elevated aMMP-8 levels in PISF and an increased risk for peri-implantitis and collagenolytic activity. **(4,5)** The Oralyzer reader, which quantitates test results within 5 min; <20 ng/ml refers to no risk of peri-implantitis and ≥20 ng/ml refers to an increased risk of peri-implantitis.

4.4 LABORATORY METHODS

Table 1. A summary of the methods used in the oral fluid-based studies I-V:

Oral fluid	Analysed protein	Methods	Study
Saliva	aMMP-8 Concentration	IFMA	I,III, IV
	MMP-8 Expression	Western Blotting	I
	MPO Concentration	ELISA	I
	PMN elastase Concentration	ELISA	I
	TIMP-1 Concentration	ELISA	I
	MMP-8, MPO, PMN, TIMP	LFQ	I
Mouth rinse	aMMP-8	PerioSafe	II, III
PISF	aMMP-8	ImplantSafe	II
	aMMP-8 Concentration	IFMA	II
	MMP-9 Activation	Gelatin-Zymography	II
GCF	aMMP-8 concentration	dip-stick	IV
	aMMP-8 concentration	IFMA	III ,V
	MMP-9 Concentration	ELISA	V
	MMP-13 Concentration	ELISA	V
	MPO Concentration	ELISA	V
	OPG Concentration	Milliplex MAP	V
	TRAP-5 Concentration	Milliplex MAP	V

GCF, gingival crevicular fluid; PISF, Peri-implant sulcular fluid; MMP-8, Matrix Metalloproteinase-8; MPO, myeloperoxidase; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase; ELISA, Enzyme-Linked Immunosorbent Assay; LFQ, label-free quantitative proteome; IFMA, time-resolved immunofluorometric assay; TIMP, tissue inhibitor of matrix metalloproteinases; PMN elastase, polymorphonuclear leukocyte elastase.

4.4.1 Immunofluorometric assay (IFMA) (I, II, III, IV, V)

A time-resolved immunofluorometric assay (IFMA) determined the active MMP-8 concentrations. The catching and tracer antibodies that were used were monoclonal MMP-8-specific 8708 and 8706 antibodies, which were supplied by (Medix Biochemica Ldt, Espoo, Finland) (Hemmilä et al.1984). The assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $CaCl_2$ 50 µM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/l diethylenetriaminepentaacetic acid (DTPA). Once diluted in the assay buffer, the samples were incubated for one hour. Thereafter, they were incubated for one hour with a tracer antibody, labelled with Europium, followed by the addition of an enhancement solution. An EnVision 2105 multimode plate reader (PerkinElmer Finland, ex. Wallac, Turku,

Finland) 1234 Delfia Research Fluorometer (Wallac, Turku, Finland) was used to measure fluorescence after five minutes. The detection limit for the assay is 0.08 ng/ml (Hemmila et al. 1984, Hanemaaijer et al. 1997).

4.4.2 Enzyme-linked immunosorbent assay (ELISA) (I, V)

The quantification of the chosen protein concentration was carried out using ELISA. In our studies, we used the following commercial ELISA kit for the saliva and GCF quantification according to the manufacturer's guidelines on diluted samples:

In study I, we used saliva samples:

- TIMP-1 (Amersham Biosciences Ltd, Buckinghamshire, UK)
- MPO (Immundiagnostik AG, Bensheim, Germany)
- PMN elastase (Bender Med Systems mbH, Vienna, Austria)

In study V, we used GCF samples:

- MMP-9, MMP-13 (Human Biotrak, Healthcare and Amersham, Little Chalfont, UK)
- MPO (Immundiagnostik AG, Bensheim, Germany)
- OPG and TRAP-5 (Milliplex MAP multiplex assay panel, Darmstadt, Germany)

The main procedure was that concentrations were measured using pre-coated wells (96-well microplates) by adding samples, standards and controls at room temperature for an incubation time of 1-2 h depending on each protocol of the shaker. Thereafter, aspiration and standard washing was carried out 4 times with wash buffer, unbound protein was wiped away and the bound protein was identified by pipetting a peroxidase-conjugated secondary anti-antibody (specific towards the desired protein). This was followed by, 1 h incubation time on the shaker, and aspiration and washing 4 times with a wash buffer. Subsequently, a TMB substrate, which was developed using hydrogen peroxide and tetramethylbenzidine, was placed on the shaker for 30 minutes. The corresponding solution was added to all the wells to stop the reaction. A wavelength of 450 nm was used to measure the optical density with a Victor X4 Multilabel Reader (PerkinElmer Finland Oy, Turku, Finland). Based on measured standards, the calibration curve was drawn and used to calculate the concentration of the measured protein. The detection limit was agreed on the basis of each Elisa kit used.

The other ELISA kit was used to detect OPG and TRAP-5 in a quantitative sandwich-based multiplex ELISA array according to the manufacturer's instructions. Briefly, a Luminex platform was used to inspect data. The basis of this quantitative immunoassay is the utilisation of colour-coded magnetic microspheres with fluorescent dyes; each dye has been coated by a specific capture antibody. Following the bead capturing an analyte, a biotinylated detection antibody is added, which is then incubated with the reporter molecule, streptavidin-phycoerythrin (PE) conjugate. The microspheres then transit through a laser;

this excites the internal dyes that mark the microspheres, while a further laser excites the PE, which is the fluorescent dye on the reporter molecule. Lastly, each microsphere is identified by signal processors that quantify the outcome using the fluorescent reporter's signals that utilise a standard curve.

4.4.3 Gelatin substrate zymography (II)

In study II, gelatin-substrate zymography was used with PISF samples to analyse gelatinolytic activity, particularly for MMP-9. Kupai et al. (2010) noted that this is based on the molecular weight to differentiate the latent and active forms situated in the different molecular weights. The technique used is based on sodium dodecyl sulfate and polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, samples were mixed with Laemmli's buffer with a 2 h incubation time and without any reagent reduction. The enzymes were denaturated and inactivated by the SDS treatment buffer. After that, to renaturate the enzyme, the gels were then washed two times for 30 min with 50 mM Tris-HCl buffer solutions. The solutions had a pH 7.5, containing 2.5% Tween 80 and 0.02% NaN_3. Furthermore, the second wash was mixed with 0.5 mM $CaCl_2$ and 1 μM $ZnCl_2$. Finally, the gels were incubated overnight at a temperature of 37°C in a 50 mM Tris-HCl buffer, pH 7.5, containing 0.02% NaN_3, 0.5 mM $CaCl_2$, and 1 μM $ZnCl_2$. The gels were then stained with 1% Coomassie Brilliant Blue R-250. MMP activity could be detected as digested zones in the gel, and gelatinolytic activity intensities were quantified using a Bio-Rad Model GS-700 imaging densitometer.

4.4.4 Western immunoblotting (WB) (I)

Immunoblotting is a frequently used laboratory method to identify and separate proteins based on molecular weight forms by using a specific antibody. The enzyme of particular interest in this study is MMP-8. The sample, in this instance, was mixed with Laemmli's buffer without any reagent reduction, and boiled for three to five minutes. Then, by type, samples were pipetted to polyacrylamide gels. During gel electrophoresis, the protein in our saliva samples were separated on the basis of molecular weight. After that, the protein were transferred to a nitrocellulose membrane and 5% skim milk in Tris-buffered saline, and 0.1% Tween 20 and 0.05% Triton X-100 were used to block the non-specific binding site of the membrane for one hour. This was followed by four 15-minutes washes, making a total of one hour. The membrane was then incubated overnight at room temperature on a shaker with primary antibody labelling specific to MMP-8. Only the antibody bound to the membrane was left after washing four more times, each of 15-minute duration, making a total of one hour. The secondary antibody was then added for one hour. The secondary antibody is conjugated to an enzyme or other material that enables the protein of interest to be detected and to appear as a band on the blot. The amount of protein present is indicated by the thickness of the band. The film is then developed to find the bound antibodies. A Model Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) was used to evaluate the intensity of different MMP-8 molecular weight forms.

4.4.5 Proteomics

The term of 'proteome' is used in the field of mass spectrometry (MS) -based proteomics to show the currently unachieved goal of identifying all proteins of a species (Beck et al. 2011). Proteomics, common in medicine and dentistry, is the study of proteomes, which is the entire set of proteins that is, or can be, expressed by a genome, cell, tissue, or organism at a certain time. Technological advances include the study of saliva as a non-invasive means of analysing systemic health. Over 5,000 proteins can be identified through saliva. However, progress in characterising and validating them further is hampered by the paucity of antibodies, which have high specificity for all of them (Beck et al. 2011, Megger et al. 2013, Bostanci et al. 2017, 2018).

Despite the success of un-targeted proteomics in identifying several different potential biomarkers, to date, none have been found applicable for clinical application for periodontal diagnostics purposes. However, available tools have identified several candidate markers that are better characterised and utilised. To date, the main source for protein quantification using antibodies to specifically target a protein of interest, is by means of antibody-based immunoassays (Aebersold et al. 2003, Bostanci et al. 2017, 2018).

4.4.6 Label-free quantitative proteomics method (I)

The following elemental stages are included in label-free quantitative proteomics. Firstly, sample preparation, which includes protein extraction, reduction, alkylation and digestion; then, sample separation by liquid chromatography (LC) or two-dimensional liquid chromatography (LC-LC), and analysis by tandem mass spectrometry (MS/MS); and finally, data analysis including peptide/protein identification, quantification and statistical analysis. Each sample is prepared separately for the label-free quantitative method. Thereafter, they are subjected to individual LC-MS/MS or LC/LC-MS/MS runs. Normally, there are two categories of measurement for protein quantification. These are ion intensity change measurements, such as peptide peak areas or peak heights in chromatography, and spectral counting of identified proteins after MS/MS analysis (Bostanci et al. 2010, 2013, Megger et al. 2013).

4.5 STATISTICAL ANALYSIS (I, II, III, IV, V)

In study I, concentrations of salivary MMP-8, MPO, TIMP-1 and PMN elastase were corrected for total protein levels. Graph Pad Prism 7 (Graph Pad Software, Inc., La Jolla, CA, USA) was utilised for the statistical analyses. Any relationships between LFQ proteomics and IFMA or ELISA data were identified for each analyte using Pearson's correlation test, which is based on log2-fold changes between all different time points. The Friedman test was used to investigate how analyte levels varied over the 35 days and differences in these levels at different time points were measured with Dunn's Multiple Comparison Test. Mixed-effects multilevel regression analyses were carried out with STATA 14.2 (StataCorp)

software in order to gain approximate values for differences in MGI and TQHPI over the 35 days. P values < 0.05 were considered statistically significant.

In study II, we used (Wilcoxon's rank sum test) to measure the significant differences and associations between two variables; the two variables are aMMP-8 levels in peri-implantitis sites measured with the ImplantSafe test and IFMA.

In study III, the correlation between the two variables was measured with the Kruskal–Wallis test ($p < 0.01$) and Spearman's test=1. The Dunn-Bonferroni test and Dunn's post-hoc test ($p < 0.05$) were also utilised. Dunn's test was used to specify significant means, while Dunn's Multiple Comparison Test specified significant differences between 3 or more means. The Dunn-Bonferroni test can be used to study associations between variables and also to make comparisons between the participant groups at baseline.

In study IV, G power analysis of MMP-8 biomarker values was used to work out a minimum specimen size, accepting a power of 85% and a p value of < 0.05. The non-parametric Kruskal-Wallis test, conducted for independent specimens, was carried out to work out the statistical dissimilarities among the subject groups. The aMMP-8 test's precision was assessed through receiver operating characteristic curve (ROC) analysis. The Kappa statistic was utilised to find concurrence among aMMP-8 quantitative determination and the result from the test stick. Statistical significance was set at a level of 5%, and this was the marker for all analyses that utilised the SPSS 22.0 statistical software program (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

In study V, sample size calculations were carried out before the study began in order to determine the minimum number of participants. A 0.5-mm mean difference between whole-mouth PD values of SDD and placebo groups was considered, and it was assumed that the standard deviation in both groups would be 0.5. With a power of 85%, p value = 5%, normal distribution, and equal variances in the placebo and SDD groups, it was calculated that the minimum sample size required was 14 participants per group. PD was chosen as the primary outcome variable, with the secondary outcome variable being GCF biomarker levels, and the unit of analysis being a patient. The mean values of whole-mouth clinical parameters and the mean values for clinical parameters per patient were averaged. The Friedman test was employed to make intragroup comparisons of the clinical parameters as recorded at the baseline and during the 3, 6, 9, and 12 months evaluations. The Bonferroni-corrected Wilcoxon-signed rank test was used to assess the significance of changes, while significant differences between the two participant groups were assessed with the Mann-Whitney test. Web-based software (R software, version 3.3.1, package: nparLD, R Foundation for Statistical Computing, Vienna, Austria) was used to test biochemical variables with the non-parametric Brunner-Langer model.

5. RESULTS

5.1 ASSOCIATION BETWEEN LFQ PROTEOMIC AND CONVENTIONAL IMMUNOASSAY IN EXPERIMENTAL GINGIVITIS STUDY (I)

5.1.1. Evaluation of clinical parameters

The current study included ten participants who had taken part in the original study (Nascimento et al. 2019). They consisted of four males and six females aged between 21-24 years. Collected samples and data indicated TQHPI and MGI scores (n = 50) in the induction and resolution phases.

Table 2. Mean and (± SD) for TQHPI and MGI.

	Day 0	Day +7	Day +14	Day +21	Day + 35
TQHPI	0.1 ± 0.1,	1.3 ± 0.2	2.2 ± 0.4	2.9 ± 0.4	0.6 ± 0.3
MGI	0.0 ± 0.0	0.5 ± 0.1	1.3 ± 0.3	1.9 ± 0.1	0.3 ± 0.4

During phase I, days 0-21, there were continuous increases in both TQHPI and MGI scores. However, by the end of R-phase, days +21 - +35, these had reverted to levels similar to baseline. Mixed-effects multilevel regression analyses were used to compare all of the different time points. Over time, there were notable differences for both TQHPI ($p < 0.001$) and MGI ($p < 0.04$). Table 2.

5.1.2 Evaluation of MMP-8 salivary level concentration and expression by LFQ, IFMA, and WB.

In an effort to assess their ability to reflect plaque accumulation and gingival inflammation development over time, LFQ proteomics, WB and IFMA were used to measure MMP-8 salivary level concentration and expression. Readings were taken at baseline (day 0), during the plaque accumulation phase (day 0 to day +21), *i.e.*, l-phase, and following the plaque removal phase (day +21 to day +35), i.e., R-phase.

It was observed that by using the LFQ proteomics method there were notable differences in salivary MMP-8 over time ($p < 0.01$), which were identified by the Friedman test. In particular, major MMP-8 differences were observed between day +7 and day +21 ($p < 0.05$), and day +7 and day +35 ($p < 0.05$). On the other hand, the result we obtained in the Friedman test when we used the IFMA method displayed no notable differences over time ($p > 0.05$). The expression of different molecular weight forms of MMP-8 was analysed by WB. It is evident that during the development and resolution of gingival inflammation (day 0 to +35), there is latent full-size pro-form MMP-8 expression (proMMP-8: 70-80 [kDa]). Discounting their overall MMP-8 concentrations at each point in time, neither sample

showed any activated or fragmented MMP-8 (aMMP-8: 50–60 kDa and <45 kDa forms) respectively.

5.1.3 LFQ proteomic and ELISA methods to evaluate salivary levels of TIMP-1, MPO, and PMN elastase

ELISA and LFQ proteomics methods were used to measure (n = 50) TIMP-1, MPO, PMN elastase. Measurements were taken at baseline (day 0), at day 0 to +21, l-phase, during plaque accumulation, and at day +21 to +35, R-phase, following plaque removal.

Notable differences were observed in the Friedman test, measured by LFQ proteomics, in TIMP-1 and PMN elastase salivary levels over time (both $p < 0.05$). In TIMP-1, in particular, there was a noticeable difference between day +7 and day +35 ($p < 0.05$). Whereas, in comparison, between day 0 and day +35, there was uncertain significance ($p > 0.05$). When using LFQ proteomics to measure MPO ($p > 0.05$), no significant variations were observed over time.

5.1.4 Connection between LFQ proteomics and IFMA or ELISA

To establish the association between conventional antibody-based methods and antibody-independent discovery proteomics, MMP-8 was used as the gingival inflammation model marker. There was a definite association for salivary MMP-8 levels between LFQ proteomics and IFMA ($p < 0.001$) with a Pearson's correlation coefficient (r of 0.36) resulting from time-independent correlation analysis. Further results showed a positive association for MPO ($r = 0.39$, $p < 0.0001$) and PMN elastase ($r = 0.33$, $p < 0.001$), following further analyses between ELISA based on TIMP-1, MPO and PMN elastase and LFQ proteomics, while TIMP-1 ($r = -0.24$, $p < 0.05$) showed a negative association.

5.2 POTENTIAL OF POC TESTS (PERIOSAFE AND IMPLANTSAFE WITH ORALYZER READER) IN DETERMINING PERIODONTAL AND PERI-IMPLANT DISEASES (II)

5.2.1 PoC chairside tests in diagnostic applications and responding to treatment outcomes

The healthy control subjects were periodontally and systemically healthy dental students n=10, and the other n=10 were clinically diagnosed, with X-ray confirmation, to all being adult chronic periodontitis patients. A PerioSafe visual test confirmed that all 10 patients were aMMP-8 positive. Oralyzer readers quantitated their aMMP-8 lateral-flow test-sticks before (all ≥20 ng/ml, visually [+]) and after periodontal treatment, SRP and anti-infective treatment. The bleeding on probing and pocket depths were reduced with SRP and anti-infective treatment. Levels of aMMP-8 in mouth rinse, which were based on visual assessments of the test results, changed from positive (+) to negative (−) as a result of SRP.

Oralyzer reader quantitation confirmed this by recording readings from ≥20 ng/ml (positive [+]) to <20 ng/ml (negative [−]).

In the same study, the experiment showed that in 5 minutes peri-implantitis sites (n = 29) were detected in the ImplantSafe aMMP-8 PoC/chairside sulcular fluid test sites. This allows treatment to be monitored as the test differentiates them from clinically healthy peri-implant sites (n = 32). Clinical and X-ray diagnoses were conducted to identify peri-implantitis and healthy sites. Elevated aMMP-8 levels were detected in all peri-implantitis sites (29 = 100% ImplantSafe-positive ≥20 ng/ml [124,60 ± 22.50 ng/ml]) . This differed from the low aMMP-8 levels (32 sites/<20 ng/ml [18.60 ± 3.46 ng/ml]) present in all clinically healthy sites, all of which were ImplantSafe PoC-negative. This difference was statistically significant (p < 0.0001 Wilcoxon test). Treatment applied to peri-implantitis sites resulted in positive ImplantSafe tests becoming negative. The sensitivity and specificity for ImplantSafe tests are 100%, which was clearly demonstrated in the results of this pilot case-control study. The quantitated PoC tool, the Oralyzer reader, successfully showed positive results.

In the same way, the IFMA method was used for quantitative analysis of aMMP-8 from PISF, and the quantitated gelatin zymography method was used for the densitometrical analysis of all forms of MMP-9 with the same samples of PISF. The IFMA results was correlated with ImplanSafe test results, and positively differentiated implant health and disease. On the other hand, gelatin zymography results indicated that it was not possible to differentiate peri-implant health and disease with any form of MMP-9 or total MMP-9.

5.3 UTILISATION OF AMMP-8 TEST AS A BIOMARKER IN NEW PERIODONTAL CLASSIFICATION (III, IV)

5.3.1 Performance of aMMP-8 tests analysed by quantitative chairside mouth rinse tests (PerioSafe and Oralyzer) (III)

Table 3. Categorisation of 150 Greek adults according to the new periodontal classification system

Sex	Healthy	Stage I	Stage II	Stage III	Grade A	Grade B	Grade C
Women	11	14	39	12	12	47	6
Men	20	1	42	11	2	44	8

The results included 150 Greek adults, which were classified by the new periodontal classification system. In comparison with healthy patients, aMMP-8 levels in mouth rinse were considerably higher in patients with more severe periodontitis stages and grades (Kruskal–Wallis test, $p < 0.01$ and Dunn-Bonferroni test for pairwise post-hoc comparisons, $p < 0.05$). Among the 150 Greek patients, (stage I–III and Grades A–C) were successfully differentiated (Tonetti et al. 2018). Table 3.

5.3.2 Diagnosis and association between BOP, VPI and aMMP-8 mouth rinse levels (III)

The periodontitis stage and grade (Kruskal–Wallis test, $p < 0.01$) affected both BOP and visible plaque index (VPI) values. These two conditions are loosely associated with one another (Spearman's Rho = 0.586, $p < 0.001$). Also, BOP was more closely associated with aMMP-8 levels than plaque levels (Spearman's Rho = 0.586, $p < 0.001$; Spearman's Rho = 0.269, $p < 0.001$); respectively. The risk of increased false positive diagnosis was exhibited more in BOP and VPI than compared with the aMMP-8 mouth rinse test. Our group in Helsinki University, lead by Professor Timo Sorsa, have modified the new grading system based on the mouth rinse test (PerioSafe) and Oralyzer reader according to (Tonetti et al. 2018)'s periodontitis classification system. Table 4.

5.3.3 Efficacy of aMMP-8 tests measured with immunotest dip-stick tests of GCF samples (IV)

5.3.3.1 Assessment of clinical periodontal parameters

In study IV, the two groups with periodontitis significantly differentiated from gingivitis and healthy groups ($p < 0.05$). The gingivitis group and the healthy group had PD and CAL scores that were much lower than the stage III and stage IV groups. with statistically significant differences ($p < 0.001$). In turn, the gingivitis group's PD and CAL values were higher than the ones in the healthy group ($p < 0.05$). PI and PBI results were also much greater for stage III and stage IV groups, with gingivitis exceeding the healthy group ($p < 0.001$). The stage III and stage IV groups' location-specific PD and CAL scores were much greater than in the other two groups ($p < 0.001$). While PD and CAL outcomes were equal for the gingivitis and healthy groups ($p > 0.05$). The scale was similar for PI and PBI, with stage III and stage IV being the highest, and gingivitis surpassing the healthy group ($p < 0.001$).

5.3.3.2 Sensitivity of aMMP-8 dip-stick test

Positivity levels for the stage III group were 94.4% in regard toaMMP-8 PoC tests; they were 94.7% for the stage IV group, 81% for the gingivitis group and 18.2% for the healthy group. The aMMP-8 activity of the selected cut-off point resulted in sensitivity of 83.9% and specificity of 79.2%. The ROC curve demonstrates aMMP-8's diagnostic outcome in relation to periodontitis. aMMP-8 activity displayed accuracy within good ranges (AUC = 0.87, 95% CI 0.799-0.958).

5.3.4 Distinguishing strength of aMMP-8 identification in mouth rinse, GCF and saliva (III, IV)

In study III, the format of this study allowed for the comparison of salivary aMMP-8 IFMA measurements with aMMP-8 PoC/chairside mouth rinse tests. The same antibody was used in both methods. In a sample of Greek adults, it was found that the mouth rinse aMMP-8 measurements were more accurate than in saliva and showed no false positives. Healthy

Table 4.

EFP Grading Parameters
(modified by the University of Helsinki, Prof. T. Sorsa)*

Periodontitis grade *TONETTI ET AL.*

Periodontitis grade			Grade A: Slow rate of progression	Grade B: Moderate rate of progression	Grade C: Rapid rate of progression
Primary criteria	Direct evidence of progression	Longitudinal data (radiographic bone loss or CAL)	Evidence of no loss over 5 years	<2 mm over 5 years	≥2 mm over 5 years
	Indirect evidence of progression	% bone loss/age	<0.25	0.25 to 1.0	>1.0
		Case phenotype	Heavy biofilm deposits with low levels of destruction	Destruction commensurate with biofilm deposits	Destruction exceeds expectation given biofilm deposits; specific clinical patterns suggestive of periods of rapid progression and/or early onset disease (e.g., molar/incisor pattern; lack of expected response to standard bacterial control therapies)
Grade modifiers	Risk factors	Smoking	Non-smoker	Smoker <10 cigarettes/day	Smoker ≥10 cigarettes/day
		Diabetes	Normoglycemic/ no diagnosis of diabetes	HbA1c <7.0% in patients with diabetes	HbA1c ≥7.0% in patients with diabetes
Risk of systemic impact of periodontitis[a]	Inflammatory burden	High sensitivity CRP (hsCRP)	<1.0 mg/L	1.0 to 3.0 mg/L	>3.0 mg/L
* **Biomarkers**	**Indicators of CAL/bone loss or collagene destruction**	**Saliva, gingival crevicular fluid, serum**	**No/Slow = aMMP-8 level < 10 ng/ml (no) OR 10–19.9 ng/ml (slow)**	**Moderate = aMMP-8 level >20 ng/ml**	**Rapid = aMMP-8 level > 30 ng/ml (Status 2020) substantially elevated plus co-factors**

patients showed no positive aMMP-8 mouth rinse test results. The sensitivity of the test was comparable with an increasing number of periodontal pockets. Same results were observed in study IV, when we compare GCF with salivary MMP-8 using the IFMA method. The stage IV group's GCF aMMP-8 IFMA levels were greater than those for stage III, gingivitis and healthy groups ($p = 0.01$, $p = 0.001$, $p = 0.00$, respectively). The healthy group in turn had much lower levels ($p < 0.05$) than stage III and gingivitis groups, and the aMMP-8 IFMA levels were similar for the stage III and gingivitis groups ($p > 0.05$). While in salivary samples, stage III, stage IV and gingivitis groups had much greater levels of saliva aMMP-8 than the healthy cohort ($p = 0.00$), but there was little variation between the first three groups ($p > 0.05$).

5.4 EFFECTIVENESS OF SDD MEDICATION ON CLINICAL PARAMETERS AND GCF BIOMARKERS (V)

5.4.1 Assessment of clinical periodontal parameters

Both the demographics of patients, relating to gender, age and smoking habits, were similar in the SDD (n = 15) and placebo (n = 15) groups ($p > 0.05$). The study groups showed almost the same baseline clinical periodontal parameters ($p > 0.05$). There were noticeable improvements in the periodontal conditions of both the SDD and placebo groups between the baseline and the re-examinations at 3, 6, 9 and 12 months ($p < 0.0125$).

During the study period, there was a marked decrease in the whole-mouth CAL of the SDD group ($p < 0.0125$), in comparison with the placebo group, where there was a noticeable improvement in whole-mouth CAL scores at 3 months ($p < 0.0125$). At other time points, there were no significant changes ($p > 0.0125$) in the placebo group.

At the 6, 9 and 12-month follow-up examinations, there was more PD reduction in the SDD group than in the placebo group ($p = 0.04$, $p = 0.02$, and $p = 0.04$, respectively). However, at 3 months, both had shown similar PD reductions ($p > 0.05$). The SDD group showed a marked improvement over the placebo group in CAL, which was most pronounced at 6 and 9 months ($p = 0.04$, $p = 0.04$, respectively).

At other time points, both groups displayed similar whole-mouth CAL scores ($p > 0.05$). At 3, 6 and 9 months, the SDD group displayed significantly improved GI scores in comparison with the placebo group ($p = 0.01$, $p = 0.01$, and $p = 0.01$, respectively). Throughout the entire study period, both groups showed a significant equal reduction in their PI scores ($p > 0.05$).

5.4.2 Evaluation levels of GCF biomarkers (MMP-8, MMP-9, MPO, OPG, MMP-13 and TRAP-5)

Generally, at baseline, the SDD (n = 15) and placebo (n = 15) groups showed similar results between the mean values of the studied clinical parameters and GCF of the study sites ($p >$

0.05). At 3 months, there was a noticeable improvement, and this was continued throughout the 12-month study period in comparison to baseline ($p < 0.0125$). No noticeable difference was noted between the groups at any of the time points ($p > 0.05$).

MMP-8

There was an equivalent amount of GCF MMP-8 at baseline for both groups ($p > 0.05$). At the 12-month point, there was a marked decrease in the total amount of GCF MMP-8 in comparison to baseline ($p < 0.05$). However, at the 6-month mark, it was observed that the SDD group showed a greater decrease in comparison to the placebo group, yet the difference was marginal. The total amount of GCF MMP-8 remained stable during both groups' periods for the remainder of the study.

MMP-9

At baseline ($p > 0.05$), the GCF MMP-9 total amounts were alike both for the SDD and placebo groups. Thereafter, compared to baseline, there was a marked decrease in both groups at the 12-month point ($p < 0.05$). However, it was observed that, in comparison to the placebo group, the SDD group displayed a greater decrease in the third month. Thereafter, both groups showed a similar decrease pattern. Thereafter, even though levels were still considerably lower than the baseline levels, no more decrease was noted in either group.

MPO

There was a marked MPO decrease in both groups, and at all times, compared to baseline ($p < 0.05$). The total GCF amount recorded a decrease in the third month in comparison with the placebo group, in a similar manner to the MMP-9 pattern. Following similarities in the sixth month, the placebo group showed an increased level throughout the remainder of the study period. In the SDD group, there was a continued decrease in GCF MPO levels over the period of 12 months.

OPG

At baseline, both groups had similar total GCF OPG amounts ($p > 0.05$). This decreased considerably in both groups, when measured at all time points, in comparison to baseline ($p < 0.05$). From the third to the twelfth month, there was an increased change in the placebo group, whereas GCF OPG levels continued to decrease in the SDD group over the period of 12 months.

In a major part of the study population, both GCF TRAP-5 and MMP-13 levels remained below the detection level. Half of the population, 15 of the 30 participants, was observed to having MMP-13, and only 5 of the 30 CP patients (17%) in the baseline samples were detected of having TRAP-5.

6. DISCUSSION

This thesis has identified and presented commercially available lateral-flow immunotests based on aMMP-8 oral fluid -specific PoC, chairside, namely PerioSafe and ImplantSafe, together with the digital Oralyzer reader, for the diagnosis of periodontal and peri-implant diseases. Various oral fluids, such as saliva, GCF, PISF and mouth rinse were studied. The tests differentiated periodontal and peri-implant health and diseases in an excellent manner. In addition to this, the PerioSafe test (mouth rinse test) can be incorporated as the staging and grading biomarker in the new classification system of periodontitis. LFQ can quantify enzyme levels in saliva, significant but weak correlations between IFMA or ELISA and LFQ. The use of SDD as an adjunctive for three months to non-surgical periodontal therapy compared to non-surgical periodontal therapy alone in periodontitis patients' results in the further improvement of clinical periodontal parameters and GCF biomarkers of periodontal tissue breakdown over a 12-month period.

6.1 PERFORMANCE OF AMMP-8 AS A ROBUST BIOMARKER IN PERIODONTAL DISEASES

Periodontitis is considered to be the sixth most prevalent chronic inflammatory disease worldwide (Kassebaum et al. 2014). According to the current interpretation of etiopathogenesis, a prime issue in the incipience and development of periodontal diseases is the host immune response (Nascimento et al. 2017). Dysbiotic plaque accumulation generates a host immune-inflammatory reaction, whereby neutrophils are attracted to the infected site by chemokines and pro-inflammatory cytokines (Scott et al. 2012). Thereafter, neutrophils and other cells, including plasma and gingival sulcular epithelial cells and fibroblasts, result in the release of proMMP-8, a latent form of MMP-8 (Kinney et al. 2007). The facilitation of leukocyte migration, in particular neutrophil granulocytes, towards the periodontal sulcus by cleaving collagens and other extracellular matrix components, is the prime biological function of MMP-8 in the periodontium (Kinney et al. 2007). The destruction of inflammation-related tissues in periodontitis, and the presence of other inflammatory diseases, results from the copious and non-controlled expression, release and activation of MMP-8 together with other MMPs (Nagase et al. 2003). MPO is the main cause of oxidative burst and host-derived proteases leading to MMP-8 activation. MMP-8 can also be activated by bacteria derived from proteases, such as *T denticola* (dentilisin) or *P gingivalis* (gingipains) (Amulic et al. 2012, Gursay et al. 2018).

MMP-8 is a major subject of MMP periodontal research. There is the possibility of clinical mismanagement, albeit inadvertent, as a consequence of evidence-based knowledge paucity concerning disease progression. Diagnostic procedures are intended for the purpose of gaining pertinent information on the current periodontal disease type and level of seriousness. Enabling the verification of disease monitoring and treatment planning is required during periodontal management (Slots 2013). Advances in diagnostic research and tech-

nological development indicate that there is a likelihood of new methods being available for identifying individual periodontal risks, and the use of objective biomarkers to quantify risk (Ozmeric 2004, Taba et al. 2005).

Biomarker's research aims to identify markers for the present and future state of periodontal disease (Ozmeric 2004). Over the past 20 years, a range of immunological and microbiological methodologies have been reported as diagnostic tests. Nevertheless, it is a major requirement to ensure that the efficiency standards are set at a high level to allow periodontal diseases to use adequate, evolutionary biomarkers. Periodontitis is an infectious and inflammatory chronic disease with three stages of infection, inflammation and tissue destruction. Consequently, biomarker research aims to develop an effective biomarker that can identify at least one of these stages (Curtis et al. 1989, Kinane 2000).

Research has shown that increased levels of active MMP-8 in oral fluid, excluding its latent form, are associated with progressive clinical periodontal and peri-implant disease activity (Sorsa et al. 2006, Al-Majid et al. 2018, Leppilahti et al. 2018). Oral salivary collagenase in periodontal or peri-implant patients is found in active or activated MMP-8 form in combination with activation fragments, whereas, in an uninfected mouth, it is in latent form (Uitto et al. 1990, Sexton et al. 2011). Current and future, or developing, progressive periodontal and peri-implant disease activity can be indiscernible and subclinical. It is evident from active MMP-8 in oral fluids. MMP-8 is associated with, and precedes and predicts disease, such as CAL, active periodontal and peri-implant degeneration. A close link has been established between progressing severity of periodontal and peri-implant diseases and aMMP-8. The pronounced reduction of aMMP-8 in oral fluids results in successful periodontal and peri-implant treatment (Killi et al. 2002, Pozo et al. 2005, Sorsa et al. 2016).

Analysis of the diagnostic accuracy, namely the sensitivity and specificity of molecular biomarkers in GCF, was conducted in a recent systematic review and meta-analysis to establish the detection of periodontitis in systematically healthy subjects (Bujanda et al. 2019). MMP-8 was found to be the best performance marker, displaying a sensitivity and specificity median of 77% and 92%, respectively. This result was taken from a total of 36 potential biomarkers reported in 19 different research papers.

According to Giannobile et al. (2009), the early identification of active disease status, and the ability to monitor the response to periodontal therapy, requires the development of innovative, highly reliable, non-invasive and straightforward diagnostic methods.

6.2 PERIOSAFE AND IMPLANTSAFE WITH THE DIGITAL ORALYZER READER IN DETECTING PERIODONTAL AND PERI-IMPLANT DISEASES

The tests of a recently developed PoC system for the diagnosis of periodontitis and Peri-implantitis have resulted in promising diagnostic accuracy. The aMMP-8 test uses oral fluid. Test results verifying the diagnostic accuracy of the tests have been published for general use. The test is easy-to-use, and the system is similar to that of a pregnancy test kit (Sorsa et al. 2016, Heikkinen et al. 2016, 2017, Al-Majid et al. 2018).

The aMMP-8 tests and digital readers are simple to use, inexpensive, have high sensitivity and specificity and if used in a clinical setting, they can give rapid results with accuracies of 90% and 75-85%. The concept is innovative in that it uses a diagnostic approach to measure the active isoform of the enzyme and does not aim to measure the total MMP-8 concentration. The aMMP-8 test focuses on identifying the destructive inflammation process by using a monoclonal antibody developed to target aMMP-8 (Sorsa et al. 2006, 2016, Al-Majid et al. 2018).

Comprehensive research has been conducted into whether oral fluids could be used to locate biomarkers that were capable of diagnosing periodontitis. A biomarker that has attracted a lot of research attention in this field is MMP-8. The early identification of pre-periodontitis and pre-peri-implantitis can signify the prevention of future periodontal and peri-implantitis destruction (Axelsson et al. 2004, Thierbach et al. 2016, Ziebolz et al. 2017). Timely identification of otherwise invisible destruction, or the inception of periodontal or peri-implant collagenolysis, will allow appropriate treatment, such as secondary prevention, together with supportive periodontal or peri-implant measures to be administered (Ebersole et al. 2015, Ramseier et al. 2016, Rathnayake et al. 2017). Several studies (Lee et al. 1995, Romanelli et al. 1999, Kiili et al. 2002, Sorsa et al. 2006) have observed and recorded that elevated levels of aMMP-8, excluding latent and total MMP-8, constitute a forerunner in periodontal destruction. These levels can distinguish between gingivitis and periodontitis, and abnormally high levels of aMMP-8 illustrate that the active period of periodontal disease has begun. It can provide patients of any age or ethnic background effective treatment (Nwhator et al. 2014, Sorsa et al. 2016, Johnson et al. 2016, Al-Majid et al. 2018, Zhang et al. 2018, Raivisto et al. 2019). The condition can be monitored for both periodontitis and peri-implantitis.

The methodology used in study II, PerioSafe and ImplantSafe, together with a digital Oralyzer reader for quantitation were used to correlate periodontal and peri-implant parameters. Pre- and post-treatment and the baseline test results showed a marked differential between healthy samples and diseases. Sensitivity and specificity were 100% for all samples in terms of positive PerioSafe and ImplantSafe tests showing up as negative. IFMA was used to measure the aMMP-8 levels in PISF; zymography methods were used to measure

MMP-9. All peri-implantitis sites displayed elevated aMMP-8 levels detected by IFMA. However, gelatinolytic activation of MMP-9 could not differentiate peri-implant health or disease. So, the results are in line with previously published literature.

An aMMP-8 test in an earlier study accurately separated adolescent gingivitis or initial periodontitis patients from participants in good health on the basis of earlier radiographic examinations (Heikkinen et al. 2016, 2017). Additionally, there was an association between positive test outcomes and participants having a genetic predisposition to periodontitis. Heikkinen et al. (2017) discovered that MMP-3 genetic polymorphism and vitamin D receptors associated with early stages of periodontitis in Finnish adolescents. PerioSafe mouth rinse tests are able to be adapted for detecting early stages of periodontitis or pre-periodontitis in adolescents with predisposed genes. Researchers have revealed that increased aMMP-8 levels have been observed among type 1 and type 2 diabetes patients (Cervino et al. 2019, Fiorillo et al. 2019). Similarly, recent research has indicated the suitability of the PoC aMMP-8 test for discerning pre-diabetes and diabetes in dental management (Grigoriadis et al. 2019).

The aMMP-8 PoC test has some limitations, including patients under the age of 15, those having active orthodontic phase treatment, pericoronitis, or a mouth ulcer. These conditions can result in the aMMP-8 PoC test not successfully identifying periodontal diseases in patients and thereby generating incorrect results. Rautava et al. (2020) conducted a recent study in which it was discovered that the oral rinse aMMP-8 test to identify periodontitis appears to work less well for Crohn's disease (CD) patients than for generally healthy people. The aMMP-8 test's sensitivity and specificity assessments for discerning periodontitis were much less effective for CD patients (60.0% and 75.0%) than fundamentally healthy people (90.9% and 80.0%).

6.3 INCORPORATING THE AMMP-8 TEST IN THE NEW PERIODONTAL CLASSIFICATION SYSTEM

The new periodontal classification system contains grading parameters that could ascertain the prospects of periodontitis amplifying. These parameters were not reliable enough to confirm with any certainty when the periodontitis active period would happen, even when taking into account risk factors, such as smoking and diabetes (Leite et al. 2018, Nascimento et al. 2018). This leads to the need stronger biomarkers to be introduced that might permit dentists to determine if the periodontitis is in its active period (continuing periodontal breakdown). Dentists would then be able to track the disease's path and choose forms of treatments that would be most effective (Tonetti et al. 2018).

Disease progression monitoring requires radiographic and visual observations together with a range of measurements, such as PD and BOP, together with attachment loss. It is a technically complex evaluation requiring a high level of specialist skills. The most evi-

dent negative predictor of periodontal disease activity is BOP. The sensitivity value of the test is low, and no evidence of bleeding indicates a lack of periodontal tissue destruction. The diagnosis of current disease activity is not indicated by diagnostic tests, such as CAL loss and PD (Buduneli and Kinane, 2011), as they are only indicators of previous tissue destruction.

Our recent study III has enabled us to demonstrate aMMP-8 levels, BOP and VPI in a sample of 150 Greek participants who have been categorised according to the new classification system. Confirmation has been achieved for the usefulness of aMMP-8 as a stage and grade biomarker in the 2018 system of classification. The outcomes have demonstrated that there is a positive connection between the acuteness of periodontal disease and its grade, which suggests to what extent the disease may amplify. The outcome concurs with prior research that has indicated that aMMP-8 is very effective at predicting periodontal breakdown (the positive predictive values are between 81.8%–94.7%) Similarly, there was also an association between degrees of BOP and VPI levels and the stage and grade of periodontitis, although there was only a small degree of correlation between the parameters of BOP and VPI. Prior research has displayed that BOP only weakly forecasts future periodontal breakdown (the positive predictive value for repeated BOP occurrence was ≤ 30%). However, the sustained non-appearance of BOP is correlated much more with periodontal stability, while the joint cut-off points of BOP and VPI are capable of generating more false positives results (Buduneli and Kinane, 2011). BOP and VPI levels had a wide range of occurrence in healthy patients as opposed to patients with disease (Stages I–III and Grades A–C). In terms of periodontitis patients, this applies as much to Grade A patients' BOP and VPI levels as it does to Grade B patients. In addition, the overlap in degrees of BOP and VPI between diverse classifications increases the possibility of false positives being diagnosed. On the contrary, aMMP-8 tests did not show similar overlapping and there appeared to be a significantly reduced chance of false positives than from either BOP or VPI. Overall, aMMP-8 test proved to be the preferred indicator for differentiating between periodontal health and disease.

Similarly, in our study IV, we have explored the possibility of GCF aMMP-8 levels being linked with the various periodontal disease stages, as well as general periodontal health, by utilising an aMMP-8 PoC dip-stick test and IFMA. The most recent categorisation of periodontitis was adopted to classify the 80 Turkish participants of this research in the various phases of gingivitis, Stage III, Stage IV and health. Our goal was to appoint aMMP-8 as the designated biomarker for this new periodontitis classification system (Papapanou et al. 2017, Tonetti, Greenwell & Kornman 2018).

The key discovery of the research has been the recognition of an association between increased GCF aMMP-8 levels and a greater intensity of periodontal disease. Crucially, if aMMP-8 is not present, but latent, pro- or total MMP-8 is, this indicates a healthy individual. The degree of the disease was linked to higher values of PD, CAL, and PI. The

diagnostic precision of aMMP-8 PoC dip-stick tests in GCF samples was indeed of a high order (87%) (AUC = 0.87, 95% CI 0.799-0.958), allowing for the differentiation of phases of periodontal disease and health provided by the aMMP-8 biomarker. Such a positive link among aMMP-8 degrees and periodontal disease phases shows how useful this biomarker is in accurately assessing periodontal disease in accordance with the new classification of periodontitis. In our study IV, our results showed that higher GCF aMMP-8 levels in the stage IV group, as well as in stage III and gingivitis groups in comparison with the healthy cohort, supports the positive role of MMP-8 in periodontal and peri-implant pathogenesis as well as its biomarker role in screening, diagnosis and prevention. Positive aMMP-8 test outcomes were much more apparent in periodontitis (nearly 95%) and gingivitis (81%) groups, compared to the healthy cohort (18.02%). The indication from this is that chairside and quantitative trials could screen, discover and distinguish sites that were healthy or had gingivitis from periodontitis locations, and therefore indicate the risk of gingivitis at an early stage. It should be stressed that aMMP-8, excluding total or latent aMMP-8, is known to be present before serious periodontal degradation and elevated levels of aMMP-8 in oral fluids. Therefore, it can also provide a warning of periodontal tissue destruction. In study I, we showed that during the induction and resolution phase of gingival inflammation, it was latent MMP-8 that was present, not active MMP-8. Previous research has reported that there are links between progressive periodontitis and active aMMP-8 in oral fluids, while in gingivitis and healthy fluid, latent enzymes were most prolific (Sorsa et al 2017, Al-Majid et al. 2018).

(Raisanen et al. 2020)'s prior research demonstrated mouth rinse aMMP-8 measurements to be more effective and have greater accuracy in discerning adolescents' predisposition to periodontal health and disease as opposed to aMMP-8 saliva tests. This process was carried out several times and further progressed in our study III and IV when we compared the aMMP-8 mouth rinse and GCF test results to aMMP-8 IFMA saliva assessments using the same antibody in each method. Mouth rinse aMMP-8 test assessments showed more accurate results and provided no false positives. On the contrary, when IFMA assessments of aMMP-8 saliva levels were carried out, they provided a less clear categorisation of periodontal health and disease. We discerned aMMP-8 mouth rinse test measurements to appear to be the best way of assessing active periodontal breakdown and the possibility of future periodontal disease. Our recommendation is that aMMP-8, together with the mouth rinse test PerioSafe with the Oralyzer reader, should form an element of the new periodontal disease classification system, so as to raise the precision of periodontal disease diagnosis and its progression.

6.4 QUANTIFYING DIFFERENT NEUTROPHIL ENZYMES IN SALIVA ANALYSED BY LFQ PROTEOMICS AND ANTIBODY-BASED ASSAYS

In the period between the late 1950s and early 1960s, the idea began to evolve that there might be a link between the proteins found in oral fluids and the presence and activity

of periodontal disease. Although proteomics have facilitated the identification and validation of a number of potential biomarkers, to date none have been discovered that can be applied to the diagnosis of periodontal disease (Bostanci et al. 2017, 2018). LC-MS-based proteomics and conventional antibody-based methods ELISA and IFMA were assessed in our present study I to find out if they would be able to provide a quantitative measure of salivary biomarkers of periodontal disease. The participants of the study had induced gingival inflammation development. The model biomarkers that were utilised included MMP-8, TIMP-1, MPO, and PMN elastase. Antibody-based immunoassays were used as the main technique for quantifying proteins that target a specific protein. In the current study, the LFQ approach successfully identified and quantified the biomarkers, which were being tested for the presence of periodontal disease. The analysis revealed a significant, albeit weak correlation, between the IFMA immunoassay or commercial ELISAs and the LFQ approach. In addition, there was positive and significant correlation between MMP-8, MPO, and PMN elastase, and LFQ proteomics and IFMA or ELISA. On the other hand, there was a negative relationship for TIMP-1 (LFQ versus ELISA). One explanation for the difference may be that PMN elastase and MPO share a neutrophil origin, whereas TIMP-1 has a fibroblastic/mesenchymal cell lineage origin, *i.e.* a non- neutrophil lineage. In the same study, WB analysis was also used to assess the molecular forms of MMP-8. This research is the first to demonstrate that MMP-8 activation does not occur during the induction and resolution phases of experimentally induced gingival inflammation; instead, it was found to be present in its latent, proMMP-8 form. This finding could be important for identifying the mechanisms and processes involved in gingivitis developing into periodontitis. One limitation of the study could be the fact that the sample size only consisted of 10 participants. However, the study results still show a significant correlation between immunoassays and LFQ proteomics in terms of the assessed biomarkers.

6.5 POTENTIAL EFFECTS OF SUB ANTIMICROBIAL DOSE OF DOXYCYCLINE THERAPY ON CLINICAL PARAMETERS AND DIFFERENT BIOMARKERS OF PERIODONTAL TISSUE CATABOLISM

One potential method for managing periodontal disease is through the modulation of the host response. The anti-microbial effect of doxycycline is based on its ability to inhibit the gingival tissues and GCF collagenase activity of chronic periodontitis. Therefore, it decreases connective tissue destruction and the resorption of alveolar bone (Golub et al. 1998, Caton et al. 2000). The use of SDD has been assessed in several studies, which have analysed SDD therapy's adjunctive effect and ability to modulate host activity. Typically, SDD therapy is prescribed for a period of 14 days to 24 months (Preshaw et al. 2004, Golub and Lee 2020). In our present study V we assessed adjunctive SDD for 3 months to determine its effectiveness on clinical parameters and GCF MMP-8, MMP-9, MMP-13, MPO, TRAP-5, and OPG levels in patients with chronic periodontitis. The study was a double-blinded, placebo-controlled, parallel-arm study. The results were evaluated in chronic periodontitis patients over

a 12-month period and compared with those obtained for patients subject to only non-surgical periodontal therapy. We found both treatments resulted in significant improvements in the management of chronic periodontitis; the adjunctive SDD was clinically the most beneficial.

In regard to reducing periodontal disease and retaining clinical attachment, the findings of the study, in which adjunctive SDD was found to be clinically more beneficial than SRP therapy alone, add to and confirm the findings of previous researchers (Walker et al. 2000, Golub et al. 2001). The current study prescribed SDD in addition to SRP over a period of three months, which is shorter than that employed in past studies (Thomas et al. 1998, Golub et al. 2001). In order to minimise confounding factors, both groups in our study had similar levels of MMP-8, MMP-9, GCF and MPO levels at baseline. When adjunctive SDD or SRP therapy had been administered, the overall GCF MMP-8, MMP-9, and MPO levels reduced noticeably. When undergoing active periodontal treatment, both groups of participants experienced reduced levels of GCF biomarkers. This suggests that being instructed on oral hygiene and receiving SRP therapy, i.e. non-surgical periodontal therapy, reduces the presence of oral bacteria. However, there was a temporary decrease in these biomarkers when SRP therapy was administered alone, while SDD achieved stable biomarker reduction over the period of 12 months. Thus, it appears that mechanical treatment was only able to achieve a temporary reduction in biomarker levels, whereas SDD stabilised them for 12 months. Nevertheless, the difference was statistically insignificant. In a short-term study carried out over two weeks, collagenase activity was seen to reduce by 60–80% with SDD. This indicated that TCs were able to reduce the severity of inflammation, which in turn prevented periodontal tissue breakdown (Golub et al. 1990, 1998). The findings of this study, our previous studies and research carried out by others, confirm that the expression of host collagenases/MMPs can be downregulated or reduced with adjunctive SDD therapy by means of a mechanism that is not related to the drug's antimicrobial properties (Preshaw et al. 2004, Golub et al. 1990, 1998). This points to a potential benefit of MMP's inhibition of doxycycline; it could constitute a safe form of therapy that does not affect the normal remodelling of connective tissue but is able to effectively reduce excessive collagenase levels. Our study was the first one to evaluate SDD's adjunctive effect for GCF bone turnover biomarkers, TRAP-5 and OPG. GCF TRAP-5 levels of the chronic periodontitis patients in this study were measured, and approximately 17% of the participants were found to have TRAP-5. In both groups of this study, the total amount of GCF OPG reduced significantly in comparison to the baseline, although it increased for the placebo group from three to twelve months. The levels in the SDD group reduced continuously over the 12 months. With SDD therapy, past research has found that host modulate therapy (HMT) treatment (non-antibiotic-dose doxycycline MMP-inhibitor) reduced the severity of chronic periodontitis, the circulating levels of CRP, TNF-α and IL-6, which are biomarkers of systemic inflammation, and MMP-8 and MMP-9. The data from our study indicates that treating patients for three months will have long-lasting beneficial effects (Salvi et al. 2005, Golub et al. 2020).

6.6 LIMITATIONS AND STRENGTHS OF THE STUDY

A limitation of the present case series study was the small sample sizes: study I, n = 50, study II, n = 80, study IV, n = 80 and study V, n= 30. Larger sample sizes would allow the findings to be more reliable. This was the first-time studies such as performed in writing with ImplanSafe and Oralyzer, so it was realistic to keep the study groups small.

A strength of our study was the large sample size in study III, n=150. Furthermore, the participants were examined by a highly trained periodontist. During the last decade, MMP-8 research has increased significantly. Professor Timo Sorsa invented the aMMP-8 test, which was improved and updated during the last several years and are accepted by US FDA nowadays. PerioSafe and ImplantSafe with the Oralyzer reader were the last updated tests for qualitatively and quantitatively measuring aMMP-8 levels in oral fluids. In my studies, we approved and confirmed the reliability and efficacy of these tests. In the same way, we demonstrated the performance of aMMP-8 as a biomarker test in grading and staging of periodontal diseases according to the new periodontal classification system. We also made comparisons with clinical periodontal examination, such as BOP and PI.

7. CONCLUSIONS

1. There was a correlation between increased aMMP-8 levels in oral fluids and radiographic and clinical parameters. The use of PerioSafe and ImplantSafe with the Oralyzer reader to analyse oral fluids proved to be highly capable of differentiating site- and patient-specifically between periodontal and peri-implant health and diseases. Therefore, they can be strongly considered to be valid diagnostic, prognostic, and preventive PoC chairside technologies for periodontal and peri-implant diseases.

2. The ImplantSafe PoC chairside test and IFMA were able to measure aMMP-8 levels and effectively differentiate between diseased and healthy samples and sites, whereas zymography method targeting could not differentiate all or selective forms and total MMP-9.

3. Following the above mentioned, we can confirm that the aMMP-8 mouth rinse test can be employed as an adjunctive tool for diagnosis in order to identify or help to prevent periodontitis. It enables the application of required biomarkers in the classification of disease by stage and grade. Additionally, active ongoing periodontal breakdown can be assessed quantitatively at the point-of-care in a very short time, approximately five minutes. In general, our results indicate that it will be beneficial to incorporate aMMP-8 as the biomarker needed and wanted in the new (Tonetti et al. 2018) periodontitis classification system. This test may be a worthwhile adjunctive tool for diagnosis and prevention, assisting traditional approaches in the identification of periodontal disease.

   A further reason to encourage its use is that it does not lead to an increase in false positive results, unlike BOP and PI. It is also anti-invasive and never causes bacteraemia.

4. The results of our studies III and IV revealed that the aMMP-8 measurements from mouth rinse and GCF are more accurate for the identification of periodontal diseases and health than those taken from saliva. When saliva was used as a matrix for periodontitis biomarkers, the results were inexact and erroneous.

5. LFQ proteomics have been shown to be able to accurately quantify and identify the biomarkers in saliva. Furthermore, this method was able to measure these marker levels during the accumulation of plaque, thus showing changes caused by gingival inflammation. The fact that the correlations between the immunoassays tested here and LFQ were weak leads to a call for strategies that employ "anti-peptide" antibody development, with the latest targeted proteomic approaches based on LFQ (Bostanci et al. 2018). It is hoped that this will result in more reliable screening platforms being designed.

6. Our results suggest that providing SDD adjunctive therapy for patients with chronic periodontitis for three months together with non-surgical periodontal therapy, in comparison with non-surgical periodontal therapy alone, leads to an improvement in clinical periodontal parameters and GCF markers of periodontitis over a period of twelve months. It is likely that adjunctive SDD therapy's benefits are linked to decreased levels of two MMPs associated with periodontitis, *i.e.*, MMP-8 and MMP-9, and their potential oxidative activator MPO.

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