Risk factors and active matrix metalloproteinase-8 (aMMP-8) diagnostics for initial periodontitis in adolescents

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DOCTORAL DISSERTATION

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Once you choose hope, anything's possible.
-Christopher Reeve-

To my family

ABSTRACT

Periodontitis (gum disease) is a chronic multifactorial infectious and inflammatory disease that causes the destruction of the supporting tissues of the teeth but can also cause chronic low-grade inflammation. Thus, periodontitis has also adverse systemic effects and is associated with several underlying health conditions, including diabetes, cardiovascular diseases, chronic obstructive pulmonary disease, hypertension, and cancer. It should be also noted that periodontitis is a globally major oral disease whose incidence increases significantly between the ages of 20 and 40. These points underscore the importance of identifying the at-risk patients as early as possible for well-timed and effective prevention and treatment. Unfortunately, identifying initial periodontitis is a challenge in primary care. Often these patients are identified after the periodontal disease has already initiated, progressed and caused visible clinical signs and radiographical findings. Patients themselves usually have not been aware of the disease and its development, which is why the prevention and treatment of periodontitis are usually mostly reactive. With this background this thesis investigated new ways to improve the diagnosis of initial periodontitis.

Risk factors for periodontitis have been studied to be able to construct successful intervention strategies. The studies have mostly concentrated on adult populations, but previous studies in adolescent populations have found several risk factors, such as, smoking, diabetes mellitus, periodontopathogenic bacteria as well as social and psychological factors. Oral fluid biomarkers have also been researched extensively to find useful biomarkers to improve diagnostic accuracy in the detection of periodontitis, its real-time disease activity and in monitoring the response to periodontal therapy. Matrix metalloproteinase-8 (MMP-8), also known as collagenase-2 and neutrophil collagenase, is the main collagenase that causes tissue destruction in the gingival tissues by breaking down its main structural component collagen. Elevated MMP-8 activity in the periodontium reflects ongoing active collagenolysis and is associated with the increased active MMP-8 (aMMP-8) concentrations in oral fluids (saliva, mouthrinse, and gingival crevicular fluid). Currently, there exists both laboratory methods, such as time-resolved immunofluorometric assay (IFMA) and enzyme-linked immunosorbent assay (ELISA), but also commercially available aMMP-8 point-of-care (PoC) oral fluid tests that analyze the aMMP-8 concentrations in oral fluids. They have been suggested for monitoring the risk for periodontal breakdown and progression of periodontitis.

The aim of this research was to investigate potential risk factors and diagnostics for initial periodontitis in adolescents (Study I) and seek to further validate the preliminary results on the benefits of the aMMP-8 mouthrinse PoC testing in identifying adolescents at risk of initial periodontitis (Study II-

IV). This thesis study utilized two samples of Finnish adolescents aged 15–17 years (birth cohorts) collected in the city of Kotka in 2004-2005 (N = 501) and 2014-2015 (N = 47). In studies I and II, this thesis found that the main risk factors for initial periodontitis in adolescents were elevated aMMP-8 levels, accumulation of root calculus particularly together with elevated levels of bleeding on probing, pack-years of smoking, and male gender. Obesity and underweight were also associated with initial periodontitis and its extent. The two red complex bacteria, *Treponema denticola* (*T.d.*) and *Tannerella forsythia* (*T.f.*), were also included in the variable selection process to the ordinal logistic regression model, representing the dysbiosis of bacterial biofilm. Furthermore, aMMP-8 PoC mouthrinse test showed better accuracy in the identification of clinical signs of initial periodontitis (periodontal pocketing, at least 4mm) than BOP levels. The association of the aMMP-8 PoC test was 2.8–5.3 times stronger (in terms of odds ratio) compared with BOP 20% (full-mouth BOP percentage with cutoff of 20% for positives). Furthermore, the sensitivity of the aMMP-8 PoC test was at least 2 times higher compared with BOP 20%, while the specificity was nearly the same for both. The results suggest that utilizing aMMP-8 test instead of BOP 20% could reduce the risk of undertreatment of patients. In that regard, study III showed that patient's bacterial challenge (dental plaque and calculus) seemed to have a major impact on BOP levels compared with other factors (the extent of periodontal pocketing, aMMP-8 levels, smoking, toothbrushing and gender). If patient's oral hygiene was on a good level, BOP levels could be low even if there were several at least 4mm periodontal pockets. BOP levels seem to reflect the extent of bacterial challenge and gingival inflammation, and much less the severity of the periodontal inflammatory condition. Thus, BOP levels may offer unreliable information about the periodontal treatment need in adolescents, in case the factors affecting bleeding on probing propensity are not carefully considered. Finally, study IV suggested stronger association between initial periodontitis and aMMP-8 levels in mouthrinse than saliva. Thus, mouthrinse seems preferable oral fluid in aMMP-8 diagnostics between the two.

In conclusion, this thesis study showed that aMMP-8 PoC mouthrinse test could be a useful tool in periodontal diagnostics to improve the detection of adolescents at risk of initial periodontitis and targeting their disease prevention. On the long term, using aMMP-8 test could potentially help to improve the effectiveness of prevention and reduce the future adverse effects of periodontitis and the need for expensive dental specialist treatment among the at-risk patients. This could reduce the health and economic burdens for these individuals, communities and society caused by periodontitis. Finally, the results in this thesis offer several interesting research questions for future follow-up studies on initial periodontitis in adolescents.

TIIVISTELMÄ

Parodontiitti eli hampaan kiinnityskudossairaus on krooninen monitekijäinen infektio- ja tulehdussairaus, joka aiheuttaa hampaiden tukikudosten tuhoa. Sillä on myös haitallisia systeemisiä vaikutuksia. Kroonisena matala-asteisena tulehdustilana parodontiitti on yhdistetty useisiin kroonisiin sairauksiin, mukaan lukien diabetes, sydän- ja verisuonitaudit, keuhkoahtaumatauti, verenpainetauti ja syöpä. Parodontiitti on maailmanlaajuisesti merkittävä suun sairaus, jonka ilmaantuvuus lisääntyy merkittävästi 20–40-ikävuoden välillä. Nämä seikat korostavat nuorten riskipotilaiden tunnistamisen tärkeyttä mahdollisimman varhaisessa vaiheessa, jotta parodontiitin ennaltaehkäisy ja hoito olisi oikein ajoitettua ja tehokasta. Siten voitaisiin vähentää parodontiitin aiheuttamia terveydellisiä ja taloudellisia rasitteita niin yksilöille, yhteisöille kuin yhteiskunnallekin. Valitettavasti alkavan parodontiitin tunnistaminen on haastavaa perusterveydenhuollossa. Usein nämä potilaat tunnistetaan vasta sen jälkeen, kun parodontiumissa kiinnityskudossairaus on jo alkanut, edennyt ja aiheuttanut selkeästi havaittavat kliiniset ja röntgenologiset löydökset. Potilaat itse eivät ole yleensä tietoisia sairaudestaan ja sen kehittymisestä, minkä vuoksi parodontiitin ehkäisy ja hoito ovat yleensä lähinnä reaktiivisia. Tämän taustan pohjalta tässä väitöskirjatutkimuksessa pyrittiin tutkimaan uusia tehokkaita tapoja tukea alkavan parodontiitin diagnosointia.

Parodontiitin riskitekijöitä on tutkittu, jotta kyettäisiin muodostamaan tehokkaita interventiostrategioita. Tutkimukset ovat enimmäkseen painottuneet aikuisväestöön, mutta aiemmissa tutkimuksissa nuorillakin on löydetty useita parodontiitin riskitekijöitä, kuten tupakointi, diabetes mellitus, parodontopatogeeniset bakteerit ja sosiaaliset ja psykologiset tekijät. Lisäksi suunesteistä on tutkittu laajasti biomerkkiaineita, jotka parantaisivat parodontiitin ja sen hetkisen tautiaktiivisuuden diagnostisoinnin tarkkuutta mutta myös parodontiitin hoitovasteen seurannan tarkkuutta. Matriksin metalloproteinaasi-8 (MMP-8), joka tunnetaan myös nimellä kollagenaasi-2 ja neutrofiilikollagenaasi, on merkittävä ienkudosten päärakenneainetta kollageenia hajottava ja kudostuhoa aiheuttava kollagenaasi. Kohonnut MMP-8-aktiivisuus parodontiumissa kuvastaa aktiivista kollagenolyysiä ja on yhteydessä kohonneisiin aktiivisen MMP-8:n (aMMP-8) pitoisuuksiin eri suunesteissä (sylki, suuhuuhteluvesi ja ientaskuneste). Tällä hetkellä aMMP-8-pitoisuuksia voidaan mitata suunesteistä laboratoriomenetelmillä, kuten aikaerotteisella immunofluorometrisellä määrityksellä (IFMA) ja entsyymivälitteisellä immunosorbenttimäärityksellä (ELISA), mutta lisäksi on kehitetty kaupallisesti saatavilla olevia suunesteiden aMMP-8-pitoisuuksia mittaavia vieripistetestejä. Niitä on ehdotettu käytettäväksi parodontiumin kudostuhon ja parodontiitin etenemisen riskin seurantaan.

Tämän tutkimuksen tavoitteena oli tutkia nuorten alkavan parodontiitin mahdollisia riskitekijöitä ja diagnostiikkaa (tutkimus I) ja pyrkiä lisävalidoimaan alustavia aiempia tuloksia aMMP-8-suuhuuhde-vieritestin hyödyistä alkavan parodontiitin riskinuorten tunnistamisessa (tutkimukset II-IV). Tässä tutkimuksessa hyödynnettiin kahta suomalaisista 15–17-vuotiaista kotkalaisnuorista koostuvaa aineistoa (syntymäkohorttia), jotka on kerätty vuosina 2004-2005 (N = 501) ja 2014-2015 (N = 47). Tutkimuksissa I ja II havaittiin, että alkavan parodontiitin pääriskitekijöinä olivat kohonneet aMMP-8-konsentraatiot, hammaskivi erityisesti yhdessä kohonneen ientaskumittauksen yhteydessä mitatun ienverenvuototaipumuksen (BOP) kanssa, tupakoinnin rasiavuodet ja miessukupuoli. Liikalihavuus ja alipaino yhdistyivät myös alkavaan parodontiittiin ja sen laajuuteen hampaistossa. Kaksi punaisen kompleksin parodontopatogeenistä bakteeria, *Treponema denticola* (*T.d.*) ja *Tannerella forsythia* (*T.f.*), sisältyivät myös rakennettuun ordinaaliseen logistiseen regressiomalliin, jossa ne edustivat bakteeribiofilmin dysbioosia alkavan parodontiitin riskin taustalla. Lisäksi havaittiin, että aMMP-8 suuhuuhdevieritestillä oli BOP:ia vahvempi yhteys ja parempi tarkkuus alkavan parodontiitin kliinisten löydösten tunnistamisessa (≥4 mm ientaskumuodostus). aMMP-8 vieritestin yhteys oli 2,8–5,3 kertaa vahvempi (vetosuhteella [odds ratio] mitattuna) verrattuna BOP 20 %:iin (koko hampaiston BOP-prosentti kynnysarvolla 20% positiiviselle). Lisäksi aMMP-8 vieritestin herkkyys oli vähintään kaksi kertaa suurempi verrattuna BOP 20 %:iin, kun molempien spesifisyys oli lähes sama. Tulokset viittaavat siihen, että aMMP-8-testin käyttö BOP 20 %:n sijaan voi vähentää alkavan parodontiitin potilaiden alihoidon riskiä. Tähän liittyen tutkimus III osoitti, että bakteerialtistuksella (plakki ja hammaskivi) näyttäisi olevan suuri vaikutus BOP-prosenttiin verrattuna muihin tekijöihin (hampaiston ientaskujen esiintyvyyden laajuus, aMMP-8-konsentraatio, tupakointi, hammasharjaus tai sukupuoli). Mikäli suuhygienia oli hyvällä tasolla (pieni bakteerialtistus), BOP-prosentti voi olla pieni, vaikka potilaalla olisi useita ≥4 mm ientaskuja. Siten BOP-prosentti näyttäisi heijastavan ensisijaisesti bakteerialtistuksen ja ientulehduksen laajuutta ja vähäisemmässä määrin parodontiumin tulehdustilan vakavuutta. Näin ollen BOP-prosentti voi tarjota virheellistä tietoa nuorten parodontaalisen hoidon tarpeesta, mikäli kaikkia ienverenvuotoon vaikuttavia tekijöitä ei oteta huomioon. Lopuksi tutkimus IV:n tulokset viittasivat suuhuuhteen aMMP-8-konsentraatiolla olevan vahvempi yhteys alkavaan parodontiitin kuin syljen aMMP-8-konsentraatiolla.

Yhteenvetona tämä väitöskirjatutkimus osoitti, että aMMP-8 suuhuuhdevieritesti voisi olla hyödyllinen työkalu parodontiittidiagnostiikassa parantamaan alkavan parodontiitin riskinuorten havaitsemista ja ennaltaehkäisyn kohdistamisen tarkkuutta. Pitkällä aikavälillä aMMP-8-mittaus voisi mahdollisesti ennaltaehkäisyn tehokkuutta ja vähentää riskipotilaiden keskuudessa parodontiitin aiheuttamia haittavaikutuksia ja kalliin erikoishammaslääkärihoidon tarvetta. Näin

voitaisiin vähentää parodontiitin aiheuttamia terveydellisiä ja taloudellisia rasitteita niin yksilöille, yhteisöille kuin yhteiskunnallekin. Viimeisenä, tämän väitöskirjatutkimuksen tulokset tarjoavat useita tutkimuskysymyksiä tulevaisuuden jatkotutkimuksille nuorten alkavasta parodontiitista.

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Ismo T. Räisänen
Helsinki, Finland,
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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-IV.

I Heikkinen AM, Räisänen IT, Tervahartiala T, Sorsa T. Cross-sectional analysis of risk factors for subclinical periodontitis; active matrix metalloproteinase-8 as a potential indicator in initial periodontitis in adolescents. Journal of Periodontology. 2019;90(5):484-492. doi:10.1002/JPER.18-0450

II Räisänen IT, Sorsa T, van der Schoor GJ, Tervahartiala T, van der Schoor P, Gieselmann DR, Heikkinen AM. Active Matrix Metalloproteinase-8 Point-of-Care (PoC)/Chairside Mouthrinse Test vs. Bleeding on Probing in Diagnosing Subclinical Periodontitis in Adolescents. Diagnostics (Basel). 2019;9(1):34. Published 2019 Mar 23. doi:10.3390/diagnostics9010034

III Räisänen IT, Sorsa T, Tervahartiala T, Raivisto T, Heikkinen AM. Low association between bleeding on probing propensity and the salivary aMMP-8 levels in adolescents with gingivitis and stage I periodontitis. J Periodontal Res. 2021;56(2):289-297. doi:10.1111/jre.12817

IV Räisänen IT, Heikkinen AM, Nwhator SO, Umeizudike KA, Tervahartiala T, Sorsa T. On the diagnostic discrimination ability of mouthrinse and salivary aMMP-8 point-of-care testing regarding periodontal health and disease. Diagnostic Microbiology & Infectious Disease. 2019;95(4):114871. doi:10.1016/j.diagmicrobio.2019.114871

ABBREVIATIONS

A.a.	*Aggregatibacter actinomycetemcomitans*
Acc	Accuracy
AL	Attachment loss
aMMP-8	Active matrix metalloproteinase-8
AM	Ante meridiem
AMH	Anna Maria Heikkinen
ANOVA	Analysis of variance
AUC	Area under the receiver operating curve
BCR	B-cell antigen receptor
BMI	Body mass index
BOP	Bleeding on probing, gingival bleeding
BOP 20%	Whole-mouth BOP percentage with cutoff 20% for positives
CAL	Clinical attachment loss
CD4	Cluster of differentation 4
CD8	Cluster of differentation 8
CD86	Cluster of differentation 86
CHX	Chlorhexidine
CPITN	Community periodontal index of treatment needs
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FN	False negatives
FP	False positives
GCF	gingival crevicular fluid
H2O2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HOCl	Hypochlorous acid
IFMA	Time-resolved immunofluorometric assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-	Interleukin-
LPS	Lipopolysaccharide
MCC	Matthews correlation coefficient
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NaCl	Sodium chloride
NET	Neutrophil extracellular trap
OR	Diagnostic odds ratio
PAMP	Pathogen associated molecular pattern

PCR	Polymerase chain reaction
PD#	The number (#) of sites at least with a probing depht of ≥ 4mm
P.g.	*Porphyromonas gingivalis*
pH	Measure of how acidic/basic the liquid is
P.i.	*Prevotella intermedia*
PISF	Peri-implant sulcular fluid
PMN	Polymorphonuclear
PM	Post meridiem
P.n.	*Prevotella nigrescens*
PoC	Point-of-care
PPD	Periodontal probing depth
PRR	Pattern recognition receptor
RANK	Membrane-bound receptor activator of nuclear factor-κB
RANKL	Membrane-bound receptor activator of nuclear factor-κB ligand
RBL	Radiographic bone loss
RC	Root calculus
ROC	The receiver operating curve
ROS	Reactive oxygen species
SAAVA	N-succinyl-Ala-Ala-Val p-nitroanilide
SAW	Surface acoustic wave
SDD	Subantimicrobial-dose doxycycline
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Se	Sensitivity
SNP	Single nucleotide polymorphism
Sp	Specificity
SPSS	Statistical Package for the Social Sciences
T.d.	*Treponema denticola*
T.f.	*Tannerella forsythia*
Th cell	T helper cell
TIMP	Tissue inhibitor of MMPs
TLR	toll-like receptor
TNF- α	Tumor necrosis factor-α
Treg cell	Regulatory T cell
TREM-1	Triggering receptor expressed on myeloid cells 1
Tris HCl	Tris hydrochloride
VDR	vitamin D receptor
VPI	Visible plaque index
WHO	World Health Organization
ZnCl2	Zinc chloride

1 REVIEW OF THE LITERATURE

1.1 PERIODONTAL DISEASE DIAGNOSTICS

1.1.1 HEALTHY PERIODONTIUM

The periodontium consists of the gingiva, the periodontal ligament, the root cementum, and the alveolar bone surrounding and supporting the teeth (Newman et al., 2018). Clinically healthy gingival tissues (Figure 1) are attached tightly around the tooth and to the surrounding bone, which forms a protecting barrier against the bacterial biofilm, their products and other infectious units (Newman et al., 2018). The dentogingival junction locates between the tooth and the gingiva and has epithelial and connective tissue portions (Newman et al., 2018). The epithelial portion consists of (i) the gingival epithelium covering clinically visible gingival tissues (both the free and attached gingival tissues), (ii) the sulcular epithelium forming the soft tissue lining of the gingival sulcus (or the periodontal pocket), and (iii) the junctional epithelium attaching the gingiva to the tooth at the bottom of the gingival sulcus (or the periodontal pocket) (Newman et al., 2018). The connective tissue portion is composed primarily of bundles of a mixture of type I and III collagen fibers and attaches the soft tissues of the epithelial portion to the tooth (Newman et al., 2018).

The root cementum is the calcified avascular tissue that covers the anatomic root of the tooth. It anchors the periodontal ligament that is further attached to the inner wall of the alveolar bone (Nanci & Bosshardt, 2006; Newman et al., 2018). The periodontal ligament absorbs mechanical and masticatory forces and, thus, the width of the periodontal ligament space around teeth can diminish if tooth is not in function and increase if it is in hyperfunction (Newman et al., 2018). Periodontal ligament is constantly remodeled, and the rate of formation and differentiation of the cells in the periodontal ligament play an important role in the formation and resorption of collagen, cementum and alveolar bone (Nanci & Bosshardt, 2006; Newman et al., 2018). Similarly, the alveolar bone is continuously remodeled, but the formation of new bone and its resorption are in balance in the healthy state (Newman et al., 2018).

The epithelial barrier of the gingival sulcus is the first line of defense against microbial challenge. In that regard, the junctional and sulcular epithelium is subject to a continuous cellular turnaround and remodeling, and a constant sloughing of epithelial cells in the barrier slows down and prevents the bacterial invasion (Nanci & Bosshardt, 2006; Bosshardt, 2018). Furthermore, another important defense mechanism are neutrophils, macrophages and antibodies that are constantly released into the gingival

sulcus where they target the bacterial biofilm and are flushed out of the sulcus by the gingival crevicular fluid (GCF) (Hajishengallis & Korostoff, 2017).

There is an equilibrium in a clinically healthy state between the bacterial biofilm and the defense mechanisms of the host (Van Dyke et al., 2020). However, when the bacterial challenge persists or increases, the equilibrium can be disrupted which leads to gingivitis (Van Dyke et al., 2020). The classical clinical signs of redness, swelling and bleeding on probing of the gingival tissues are visible in gingivitis but no periodontal pocketing occurs (Figure 1). Periodontal pockets with probing depths of at least 4 mm or more are not considered normal (Armitage, 2004). Probing depths are measured with a periodontal probe as the distance between the gingival margin and the bottom of the gingival sulcus (Armitage, 2004). Periodontal health can be restored in gingivitis with proper treatment, when the bacterial challenge is removed and oral hygiene is improved appropriately (Armitage, 2004). However, depending on the host's immune-inflammatory response and susceptibility to periodontitis, and if gingival inflammation is left untreated, gingivitis may progress to periodontitis that is characterized by gingival inflammation, periodontal pocketing, and alveolar bone loss as well as potential tooth mobility in its severe stage (Figure 1).

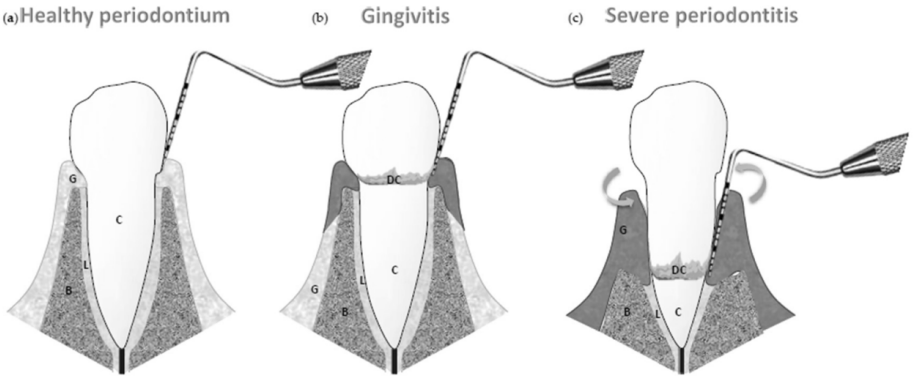

Figure 1 The structure of periodontium in (a) healthy periodontium, (b) gingivitis with plaque and calculus (DC) and a reversible gingival inflammation, and (c) severe periodontitis with gingival inflammation, periodontal pocketing, plaque and calculus, alveolar bone loss and potential tooth mobility. Periodontal structures depicted in the figure: gingiva (G), periodontal ligament (L), root cementum (C), and alveolar bone (B). Reproduced from Román-Malo and Bullon (2020) under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license https://creativecommons.org/licenses/by/4.0/.

1.1.2 CLASSIFICATION OF PERIODONTAL DISEASES

The 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions provided the latest update to the

classification/case definition system for periodontal diseases aiming to link diagnosis with prevention and treatment (Caton et al., 2018). It provides specific criteria for the following periodontal diseases, namely, (i) periodontal health and gingival health in intact and reduced periodontium (Lang & Bartold, 2018); (ii) dental biofilm-induced gingivitis and non-dental biofilm-induced gingival diseases (Holmstrup et al., 2018; Murakami et al., 2018; Trombelli et al., 2018); (iii) periodontitis (Papapanou et al., 2018; Tonetti et al., 2018); (iv) successfully treated periodontitis (reduced but healthy periodontium) and unsuccessfully treated periodontitis (persistent gingival inflammation) (Chapple et al., 2018; Lang & Bartold, 2018); (v) periodontitis as a manifestation of systemic diseases (Albandar et al., 2018; Jepsen et al., 2018); and acute lesions in the periodontium (periodontal abscesses, necrotizing periodontal diseases, and endo-periodontal lesions) (Herrera et al., 2018).

The 2017 classification system provides substantial modifications to the previous 1999 classification system (Armitage, 1999), with a focus on the definition of specific cases (Tonetti et al., 2019). Periodontal health, introduced for the first time as a case definition, is categorized into four levels: (i) an absence of pathological inflammation in a periodontium with no attachment loss (pristine periodontal health); (ii) an absence or minimal levels of clinical inflammation in a periodontium with no attachment loss (clinical periodontal health); (iii) a stable periodontal disease in a reduced periodontium (a successfully treated periodontitis patient); and (iv) a remitted/controlled periodontal disease in a reduced periodontium (a treated periodontitis patient with persistent inflammation) (Lang & Bartold, 2018). Furthermore, now the classification system recognizes three different forms of periodontitis: necrotizing periodontitis, periodontitis as a direct manifestation of systemic diseases, and periodontitis (Papapanou et al., 2018). The previously distinct diagnoses chronic and aggressive periodontitis are now under the same single description of periodontitis (Tonetti et al., 2019).

Another major change in the 2017 classification system of periodontitis is the multidimensional staging and grading system (stage I–IV periodontitis and grade A–C) (Tonetti et al., 2018). It is supposed to be utilized after a patient has been diagnosed with periodontitis (Tonetti & Sanz, 2019). The different stages of periodontitis are described by the severity and extent of the disease, and the complexity of disease management (Tonetti et al., 2018). The different grades of periodontitis assess periodontitis by its rate of progression and its future risk of progression as well as the potential risk of systemic impact related to patient's periodontitis (Tonetti et al., 2018).

Finally, the last interesting update in the 2017 classification system is that it notes the potential benefits of biomarkers for the diagnostics of periodontitis (particularly initial periodontitis) and provides a framework to incorporate biomarker(s) to the classification system in the future. Previous studies have studied extensively potential oral fluid biomarkers for monitoring disease activity and predict future disease progression of periodontitis more precisely

(Arias-Bujanda et al., 2019; Arias-Bujanda et al., 2020; Gul et al, 2020; Kc et al., 2020). Traditional methods to analyze oral fluid concentrations of biomarkers have been laboratory methods (ELISA, IFMA etc.), but currently, there also exists non-invasive point-of-care/chairside tests with a short turnaround time that are more convenient in clinical practice (Arias-Bujanda et al., 2019; Arias-Bujanda et al., 2020; Gul et al, 2020; Kc et al., 2020; Sorsa et al., 2020). A recent study utilized an aMMP-8 point-of-care test while demonstrating an association between aMMP-8 and stage and grade of periodontitis suggesting aMMP-8 as a potentially useful biomarker to be integrated into the 2017 classification system (Sorsa et al., 2020). In that regard, Heikkinen et al (2022) proposed that measuring disease activity based on biomarkers (aMMP-8) may aid in identifying potential antecedent stages before stage I periodontitis (initial periodontitis).

1.1.2.1 Case definitions of periodontal health, gingivitis, and periodontitis

In the 2017 classification system of periodontal diseases, the case definitions of i) periodontal health and ii) dental biofilm-induced gingivitis are characterized by the traditional measure for gingival inflammation, bleeding on probing (BOP), and periodontal probing depth (PPD)/ clinical attachment loss (CAL) (Chapple et al., 2018; Trombelli et al., 2018). The case definition of periodontitis relies primarily on CAL in defining a periodontitis case, however, if CAL is not available diagnostic quality radiographs to detect radiographic bone loss (RBL) are considered as an acceptable but a less sensitive substitute (Tonetti et al., 2018). CAL and RBL determine the severity and the initial stage of periodontitis that can be adjusted to higher stage according to the complexity factors, such as PPD of 4mm or more, furcation lesion, tooth mobility and masticatory dysfunction (Tonetti et al., 2018).

The case definition of periodontal health is determined by minimal levels of gingival inflammation (BOP ≤ 10%) and shallow periodontal pockets (PPD ≤ 3mm) (Chapple et al., 2018; Trombelli et al., 2018). Similarly, there are only shallow periodontal pockets (PPD ≤ 3mm) in gingivitis but the levels of gingival inflammation are elevated (BOP > 10%) (Chapple et al., 2018; Trombelli et al., 2018). Periodontitis is diagnosed primarily when interdental CAL is detectable at ≥ 2 non-adjacent teeth, or when buccal or oral CAL ≥ 3mm together with periodontal pocketing > 3mm is detectable at ≥ 2 teeth, and the observed CAL is due to periodontitis and cannot be attributed to local factors such as vertical root fracture (Tonetti et al., 2018). However, BOP is not used in the case definition of periodontitis in the 2017 classification system but for assessing gingival inflammation, diagnosing healthy periodontium or gingivitis and monitoring treated patients (Murakami et al., 2018; Tonetti et al., 2018; Trombelli et al., 2018).

Stage I periodontitis represents initial periodontitis in the 2017 classification system and is a grey area between gingivitis and periodontitis

(Tonetti et al., 2018). It is characterized by the early stages of attachment loss (CAL = 1-2 mm and maximum probing depths ≤ 4 mm) (Tonetti et al., 2018). Stage II periodontitis represents the moderate stage of attachment loss (CAL = 3-4mm), while, at stage III and IV periodontitis is more advanced periodontitis (CAL ≥ 5mm) and has already caused severe damage to the periodontal support and may cause tooth loss (Tonetti et al., 2018). Grading of periodontitis relies primarily on direct and indirect evidence of the progression of periodontitis (radiographic bone loss or CAL) (Tonetti et al., 2018). Grade A, B, and C of periodontitis represent slow, moderate and rapid rate of progression, respectively (Tonetti et al., 2018). Grade can be further adjusted to higher score if any risk factors, such as diabetes and smoking, exist in order to better estimate the future course of the disease (Tonetti et al., 2018).

1.1.3 ORAL FLUID DIAGNOSTICS FOR PERIODONTAL DISEASES

Periodontal disease diagnostics aims at identifying the signs and symptoms of the disease present in the periodontal tissues and to define its extent and severity, rate of progression as well as complexity of management (Tonetti et al., 2018). Traditional diagnosis of periodontal disease is mainly based on measuring several clinical parameters such as CAL, PPD, BOP and radiographic measurements (Kinane et al., 2017). They are being used for identifying patients with or at-risk of periodontal tissue destruction as well as planning prevention and treatment of these patients. In that regard, well-timed and accurate diagnosis is important, as the occurring periodontal tissue destruction progresses gradually and is mostly irreversible (Kinane et al., 2017). The identification is challenging especially at the initial phase of the disease when the symptoms and signs are scarce, and the disease is still painless. Thus, patients are often unaware of their disease at this point and the disease may progress to advanced stages before it is discovered and treatment can be started (Kinane et al., 2017). Moreover, the traditional clinical parameters excel in diagnosis of periodontal disease, but they are not as effective for the prediction of current and future disease activity (Kinane et al., 2017; Tonetti et al., 2018).

The identification of initial periodontitis is a challenge in general dental practice and is markedly influenced by the level of training and experience of the dentist with periodontal probing (Tonetti et al., 2018). Too often periodontal disease prevention is only reactive as the periodontal disease identified after it has already initiated and progressed with visible damage in periodontal tissues. In that regard, the future oral fluid biomarkers may improve diagnostic accuracy in the early identification of periodontitis, its current disease activity and even its initiation before its progression, while also being able to monitor its response to periodontal therapy (Tonetti et al., 2018; He et al., 2018; Gelibolian et al., 2022). Saliva, mouthrinse and gingival

crevicular fluid (GCF) provide a rich source of potential biomarkers for periodontal diseases that could reflect health and disease in the oral fluid diagnostics (Ghallab, 2018; Gul et al., 2020). The oral fluid biomarker research for periodontal diseases has been intensive during the last 30 years (Arias-Bujanda et al., 2019; Arias-Bujanda et al., 2020; Kc et al., 2020). MMP-8 is one of the most studied but also one of the most successful biomarkers for periodontitis, where the pathological elevation of its active form, aMMP-8, but not its latent form, has been shown to play an important role in the destruction of periodontal connective tissue (Lee et al., 1995; Romanelli et al., 1999; Mancini et al., 1999; Gul et al., 2020). Other promising biomarkers researched include, but are not limited to, interleukin (IL)-1β, IL-6, macrophage inflammatory protein (MIP)-1α and MMP-9 (Arias-Bujanda et al., 2020; Gul et al., 2020; Cafiero et al., 2021).

Oral fluids are readily available and can be collected non-invasively without causing bacteremia to the patients (Gul et al., 2020). Saliva and mouthrinse are collected from the whole mouth and thus can be utilized at patient-level diagnostics of the current overall inflammatory status of the periodontium. GCF on the other hand provides site-specific information from the periodontal status in the periodontal pocket (or pockets) collected from around a tooth. The advantage of collecting saliva, mouthrinse or GCF is that it is not affected, for example, by hemophiliacs or blood clotting disorders unlike collection of blood, and furthermore collecting saliva or mouthrinse is simple enough to be conducted without any medical help (Gug et al., 2019). Moreover, patients are much more willing to provide saliva, mouthrinse or GCF than blood samples, especially, if the samples are required to be collected frequently (Gug et al., 2019). Although the collection of oral fluids is relatively simple, the composition of oral fluid samples may vary depending on, for example, which collection method is used or at what temperature the samples are stored (Gug et al., 2019). Therefore, it is important to use standardized and well-defined protocols for collection, storage, and processing of all samples (Gug et al., 2019). That ensures that natural biological properties can be preserved in the samples until the biomarker analysis is performed (Gug et al., 2019).

The oral fluid biomarker detection methods currently available are either laboratory-based methods or point-of-care (POC) technologies. Laboratory methods include immunological techniques, such as enzyme-linked immunosorbent assay (ELISA), time-resolved immunofluorometric assay (IFMA) and Western blotting, separation techniques, such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the less commonly used electrochemical techniques/biosensors (Gug et al., 2019). Although they are useful for a large variety of different types of analysis, their use requires laboratory environment, skilled personnel and is time-consuming as well as expensive (Gug et al., 2019). Since the 1990s, a large number of POC technologies have been developed and researched for the diagnostics of periodontitis (He et al., 2018; Gul et al., 2020). These include microbiological test kits that detect periodontopathogenic bacteria, genetic test kits to identify

genetic susceptibility to periodontis and biochemical test kits to determine levels of bacterial and host enzymes, mediators of inflammation, and extracellular matrix components (Gul et al., 2020). The developed POC test kits can be considered as potential alternatives to laboratory methods in clinical practice for oral health practitioners, if they demonstrate high enough diagnostic sensitivity and specificity with ease of use, a rapid turnaround time, and low cost (Gul et al., 2020). Some of the biochemical test kits have shown such promising results, particularly POC oral fluid test kits based on aMMP-8 that are able to provide test results in 5-7 minutes with a great diagnostic accuracy (Gul et al., 2020).

1.1.3.1 Saliva

Saliva is a unique oral biofluid generated and secreted more than 90 % from the three major salivary glands (the parotid, submandibular, and sublingual glands) and remainder from the numerous minor salivary glands (the labial, buccal, lingual, and palatal glands) (Yoshizawa et al., 2013; Ghallab, 2018). Saliva in the mouth also contains components derived from non-salivary origin such as blood cells (neutrophils) from gingivae/GCF, small amounts of blood and tissue fluid proteins mainly from the gingivae/GCF, epithelial cells shed from mucosal surfaces, bacteria and bacterial products, viruses and fungi, and food debris (Proctor, 2016; Ghallab, 2018). Overall, there is a rich mix of potential biomarkers in saliva. A total of at least 1166 proteins have been identified in human saliva, including host-derived enzymes, tissue-breakdown products, host-response modifiers and cytokines (Cafiero et al., 2021). More than 65 of them have been studied as potential biomarkers for periodontitis and its progression (Cafiero et al., 2021).

There are two methods to collect saliva, namely, unstimulated whole-mouth saliva (or resting saliva) by passive drooling or spitting into a sterile container, and stimulated saliva through mechanical stimulation by chewing inert materials such as paraffin (Proctor, 2016; Gug et al., 2019). Unstimulated saliva consists of mostly saliva originated from submandibular/sublingual glands (68 %) and less from parotid glands (28 %) and minor glands (4 %) (Proctor, 2016). Its flow rate is known to be affected by the circadian variation (the lowest flow rate at 6 AM and the highest at 6 PM) (Proctor, 2016). Stimulated saliva, on the other hand, is originated nearly equally from parotid glands (53 %) and submandibular/sublingual glands (46 %) and a little from minor glands (1 %) (Proctor, 2016). A challenge in saliva collection that should be controlled is that metabolite concentrations in unstimulated and stimulated saliva seem to be influenced by circadian rhythm (Kawanishi et al., 2019). Thus, unstandardized time of saliva collection among patients is a potential source of bias (Kawanishi et al., 2019). Another challenge in saliva collection is that flow rate of unstimulated saliva can be limited especially among older subjects and in those taking xerogenic medications (Proctor, 2016). Finally, unstimulated saliva is usually preferred because the production of stimulated

saliva can potentially be prone to different kinds of variations that depend on which type and intensities of reflex stimulation is in use during the stimulation (Proctor, 2016).

1.1.3.2 Gingival crevicular fluid (GCF)

GCF is a site-specific oral fluid readily available from the gingival sulcus/periodontal pocket between the tooth and marginal gingiva. It provides a rich source of potential diagnostic and prognostic biomarkers for periodontal research. Previous studies have researched more than 90 different components in the GCF for periodontal diagnosis (Loos et al., 2005). GCF composition is a complex combination of molecules originating from the blood, host tissues and subgingival biofilm including leukocytes, proteins, inflammatory mediators (e.g., cytokines and chemokines), host-derived enzymes (e.g., MMPs), tissue-breakdown products, oral sulcular and junctional epithelium cells, and bacteria from the oral biofilm plaque (Barros et al., 2016; Ghallab, 2018). Furthermore, PMNs are not only the most abundant leukocyte type (≥ 95 %), but also the primary inflammatory cell-type in the gingival crevice (Delima & Van Dyke, 2003). There are always PMNs present in the gingival sulcus, even in clinically healthy situations, but their numbers increase as the severity of the inflammatory process increases (Delima & Van Dyke, 2003).

GCF can be collected, for example, using absorbent filter paper strips, capillary tubing or micropipettes, or gingival washing methods (Ghallab, 2018). It has been shown that the volume and flow of GCF in the gingival sulcus/periodontal pocket are not static but are associated with the inflammatory situation in the gingival tissues (Barros et al., 2016). Total GCF volume in the gingival sulcus comprises of two volumes: resting GCF volume that is independent of flow coming from the periodontium into the gingival sulcus and the GCF flow that depends on the collection time and flow rate (Barros et al., 2016). GCF can be collected only in small amounts from a healthy sulcus, but the GCF volume and flow increases significantly in disease when the inflammation in the periodontium increases and the depth of periodontal pockets deepens (Barros et al., 2016).

1.1.3.3 Mouthrinse, oral rinse or mouthwash

Mouthrinse is a whole-mouth oral fluid that has been suggested to collect GCF instead of saliva to measure directly the collagenase activity and provide a perspective of whole-mouth periodontal situation (Drouin et al., 1988; Gangbar et al., 1990). GCF is a unique oral fluid providing an important window to the periodontium and the current inflammatory status, but, as a site-specific oral fluid, collection of GCF (e.g., absorbent filter paper strips) for whole-mouth analysis is slow and cumbersome (Ghallab, 2018). Collection of

whole-mouth GCF in mouthrinse can be conducted in much shorter time: patient first prerinses to remove saliva and debris from the oral cavity, and after a small wait (e.g., 30 seconds) the actual test fluid is rinsed (Drouin et al., 1988; Gangbar et al., 1990). Collecting mouthrinse this way has several advantages. Prerinsing provides patients some degree of standardization of GCF collected by mouthrinse that saliva is lacking, and it also minimizes the contamination of mouthrinse collected from saliva (Drouin et al., 1988; Gangbar et al., 1990). This ensures that the potential interference from salivary tissue inhibitors of MMPs (TIMPs) to collagenase activity analyzed from mouthrinse is minimized (Drouin et al., 1988). It is also known that collecting saliva is difficult from patients with low saliva volume and saliva flow, such as patients with xerostomia and hyposalivation, which can be overcome by mouthrinse collection (Maruyama et al., 2022). Thus, mouthrinse may provide more accurate representation of the total collagenase activity in the whole-mouth periodontal tissues than saliva.

1.2 ETIOLOGY OF PERIODONTITIS

Periodontitis is a chronic multifactorial infectious and inflammatory disease that causes destruction of the tooth supporting structures and is a major cause for tooth loss (Petersen & Ogawa 2012). It is a highly prevalent oral disease that affects approximately 10% of the global population in its severe form, i.e. more than 700 million people (Petersen & Ogawa, 2012; Kassebaum et al., 2014; Frencken et al., 2017; GBD 2017 Oral Disorders Collaborators et al., 2020). Although severe periodontitis is rare among young populations, many studies have reported chronic periodontitis and initial periodontitis among them (Albandar & Tinoco 2002; Heikkinen et al. 2008; Susin et al. 2011; Susin et al. 2014; Botero et al. 2015; Catunda et al., 2019). Periodontitis can begin already in early adolescence and the susceptibility to the onset and progression of periodontitis seems to follow to adulthood (Timmerman et al., 2000; Van der Velden et al., 2006; Susin et al., 2011; Kinane et al., 2017). Prevalence of severe periodontitis increases steeply between the ages of 20 and 40 and reaches its peak at around 40 years of age remaining at that level at older ages (Petersen & Ogawa 2012; Kassebaum et al. 2014; Frencken et al. 2017). Thus, timely identification of patients at risk of the progressing periodontitis is essential to be able to control the disease and its treatment effectively.

Risk factors play an important role aiding in disease prevention and management of periodontitis. They can be utilized in the identification of patients susceptible to development and progression of periodontitis thus requiring disease prevention measures. Nunn (2000) defines three types of causation: 1) a sufficient cause, 2) a necessary cause and 3) a risk factor. A sufficient cause is the strongest type of causation and, if present, will always result in manifestation of disease (Nunn 2000). Similarly, a necessary cause is required to be present for a disease to occur, but its presence does not

necessarily lead to disease (Nunn 2000). A risk factor is a factor that is associated with disease, with or without causality (Nunn, 2000).

Periodontitis is an etiologically complex multifactorial disease in which many factors influence its development and progression. For this reason, a sufficient cause for periodontitis is considered as a combination of necessary causes rather than a single factor (Heaton & Dietrich, 2012). For example, the presence of periodontal pathogens and the host inflammatory response can be considered as necessary causes (Colombo & Tanner, 2019; Curtis et al., 2020; Van Dyke et al., 2020; Hajishengallis et al., 2020). Their interplay results in the hyper- or hyporesponsiveness of host response that with or without sufficient resolution of inflammation disrupts the homeostatic balance between the host and the oral microbiome and eventually drives gingivitis to periodontitis (Hajishengallis et al., 2020; Loos & Van Dyke, 2020; Van Dyke et al., 2020). Hence factors that may affect the susceptibility to this imbalance are important to be considered in the etiology of developing periodontitis.

1.2.1 MODIFIABLE AND NON-MODIFIABLE RISK FACTORS FOR PERIODONTITIS

There are several factors that may affect the susceptibility to periodontitis. These factors can be categorized into modifiable and non-modifiable risk factors (Borrell & Papapanou, 2005; Van Dyke & Sheilesh, 2005; Kinane et al., 2017). Modifiable risk factors are environmental, behavioral, or acquired factors that can be controlled with proper measures (Borrell & Papapanou, 2005; Van Dyke & Sheilesh, 2005). Tobacco smoking and diabetes mellitus are the two major modifiable risk factors for periodontitis (Chapple et al., 2017; Leite et al., 2018; Sanz et al., 2018).

Smoking is known to have a negative effect on the onset and progression of periodontitis (Kinane et al., 2017; Leite et al., 2018). The exact mechanisms behind the poor impact of smoking on oral health and periodontium are still not fully explained, but smoking and its many toxic compounds are believed to affect periodontal tissue destruction on many different levels including disturbing the microcirculatory, inflammatory, and immune systems (Nociti et al., 2015). They may induce unnecessary pro-inflammatory and suppressive host immunoinflammatory responses, but also impair innate defenses against pathogens, disturb antigen presentation and alter the adaptive immune responses (Lee et al., 2012; Nociti et al., 2015). In that regard, smoking is associated with alterations in the oral biofilm compositions into a potentially more periodontopathogenic and dysbiotic direction (Brook, 2011; Kumar et al., 2011; Wu et al., 2016; Karasneh et al., 2017). Smoking can also affect negatively on immune cells, such as polymorphonuclear neutrophils, macrophages, T cells and B cells, and on immune cell counts, proliferation, and functions (Nociti et al., 2015). For example, smoking can impair polymorphonuclear (PMN) neutrophil migration and chemotaxis into the site of inflammation and their function there, which eventually leads to a reduced

immune response (Güntsch et al., 2006; Sczepanik et al., 2020). This suppression of host-defense system in smokers may be reflected in certain salivary biomarker levels that have been shown to be decreased in dose-dependent manner among smokers (Kibayashi et al., 2007; Heikkinen et al., 2010). Furthermore, smoking has been associated with the development of neutrophil hyperactivity and hyperreactivity in chronic periodontitis that increases the tissue destruction by the release of proinflammatory cytokines and the overproduction of reactive oxygen species through a respiratory burst (Hajishengallis & Korostoff, 2017; Sczepanik et al., 2020). Overall, smoking increases the risk for periodontitis by 85%, but it also associates with higher rates of progression of periodontitis in adults and adolescents (Schenkein et al., 1995; Machuca et al., 2000; Heikkinen et al., 2008; Rosa et al., 2008; Schätzle et al., 2009; Leite et al., 2018; Freitag-Wolf et al., 2019; Šutej et al., 2021). In that regard, smokers seem to have more severe stage of periodontitis with more tooth loss, attachment loss, deepened periodontal pockets and less favorable response and outcomes from periodontal therapy than non-smokers do (Nociti et al., 2015).

Diabetes, especially uncontrolled and/or undiagnosed type 1 and type 2 diabetes, as well as gestational diabetes, increase the risk for periodontitis (Sanz et al., 2018; Genco & Borgnakke, 2020). The onset of diabetes often occurs before periodontitis suggesting a mechanistic link between the two diseases (Sanz et al., 2018; Genco & Borgnakke, 2020). Furthermore, diabetes is known to have a bi-directional relationship with periodontitis and especially a poorly controlled diabetes affects metabolic control and insulin resistance and also increases inflammatory burden of diabetic patients (Preshaw et al., 2012; Sanz et al., 2018; Genco & Borgnakke, 2020; Heikkilä et al., 2022). For example, the risk for developing periodontitis has been estimated to be almost three times higher in subjects with diabetes compared with subjects with normal glucose levels (Mealey & Ocampo, 2007). Diabetes has also a negative effect on the periodontium at young age promoting periodontitis in children and adolescents (Lalla et al., 2006; Dakovic & Pavlovic, 2008; Genco & Borgnakke, 2013; Kinane et al., 2017; Genco & Borgnakke, 2020; Rapone et al., 2020).

There are also variable amount of evidence for several other modifying risk factors for periodontitis including systemic diseases (obesity and metabolic syndrome and some auto-immune diseases in addition to diabetes), socioeconomic status, microorganisms (bacteria and viruses), osteopenia/osteoporosis, HIV infection, stress levels, vitamin D, nutrition and alcohol consumption (Borrell & Papapanou, 2005; Van Dyke & Sheilesh, 2005; Genco & Borgnakke, 2013; Reynolds, 2014; Tegelberg et al., 2019; Tegelberg et al., 2021). Several systematic reviews have reported an association between periodontitis and obesity in adults, adolescents and children (Khan et al., 2018; Jepsen et al., 2020). Similar results have been reported in recent systematic reviews for the association between periodontitis and metabolic syndrome, but the evidence has been largely cross-sectional

(Abhalla-Aslan et al., 2019; Jepsen et al., 2020). A systematic review suggests that stress-related disorders such as depression may have an impact on inflammatory mediators and inflammatory responses thus increasing the risk of periodontitis and its progression (Decker et al., 2020).

The available longitudinal evidence shows that relatively low early adulthood socioeconomic status (defined by education, occupation or income) increases the likelihood of future onset and progression of periodontitis (Schuch et al., 2017; Schuch et al., 2018). Similarly socioeconomic status of the parents has been demonstrated to increase the probability of periodontal diseases in children (Tadakamadla et al., 2020; Schmidt et al., 2022). Furthermore, early evidence indicates that children exposed to passive smoking at home or having parents with periodontal diseases are more likely to have periodontal diseases themselves (Tadakamadla et al., 2020). Good oral hygiene achieved by regular toothbrushing and dental visits is an important preventive method against periodontitis (Zimmerman et al., 2015; Lertpimonchai et al., 2017). However, it has been estimated that dental plaque has overall 20% impact on the direct risk for developing periodontitis, which underscores the importance of not neglecting other modifying factors in the prevention of periodontitis (Lang & Bartold, 2018).

Oral microbiome dysbiosis and outgrowth of periodontopathogenic bacteria in subgingival biofilm, such the red complex triad *Treponema denticola*, *Porphyromonas gingivalis* and *Tannerella forsythia*, have shown association with periodontitis (Curtis et al., 2020). As the bidirectional balance between the subgingival microbiome and host's inflammatory and immune response disrupts, dysbiotic changes follow in the microbiome, which increases the expression of virulence factors and inflammatory responses leading to periodontal tissue destruction (Curtis et al., 2020).

Non-modifiable factors are individual characteristics that one cannot or has difficulties to change such as age, gender, race/ethnic background, genetic factors and gene polymorphisms (Borrell & Papapanou, 2005; Van Dyke & Sheilesh, 2005). Ageing is associated with increases in the risk of chronic diseases such as periodontitis (Reynolds, 2014). As discussed earlier in this section, the prevalence and severity of periodontitis increases with age (Petersen & Ogawa 2012; Kassebaum et al. 2014; Frencken et al. 2017). In that regard, elevations in systemic markers of inflammation associated with ageing have been reported (Singh & Newman, 2011). However, ageing seems to have a small influence on the subgingival biofilm composition and mainly Actinomyces species are present in higher levels and proportions in older adults compared with young adults or adults (Feres et al., 2017).

Men have been documented to have a higher prevalence of periodontitis compared with women (Borrell & Papapanou, 2005; Kassebaum et al., 2014; Frencken et al. 2017). Socioeconomic and behavioral factors as well as differences in the access to oral care, but also biological factors such as sex specific hormonal, genetic and epigenetic factors affecting immunity may offer a possible explanation to the difference between men and women (Ioannidou,

2017). Yet, more research is still needed in this area. Moreover, differences in the risk of developing periodontitis have been reported between different racial and ethnic groups (Albandar, 2008). The race-ethnicity factor may be linked to behavioral and socioeconomic factors but biological/genetic predisposition seems a possible explanation, as well, especially regarding the prevalence of aggressive (rapidly progressing) periodontitis (Albandar, 2008; Susin et al., 2014; Bouziane et al., 2020). For example, significant epigenetic changes in the DNA methylation status have been found in African-American children and adolescents with localized aggressive periodontitis compared with healthy controls (Shaddox et al., 2017).

Previous studies have also found that genetic predispositions may contribute to the risk of onset and progression of periodontitis in adults and adolescents (Loos et al., 2015; Heikkinen et al., 2017a; Heikkinen et al., 2017b; Loos & Van Dyke, 2020). The impact of genetic factors and gene polymorphisms may be as large as 33–50% of the overall susceptibility to periodontitis (Chapple et al., 2017; Loos & Van Dyke, 2020). Furthermore, the genetic contribution to periodontitis seems to be larger in young populations compared with older subjects (Loos & Van Dyke, 2020). Genetic changes can be inherited but several factors during a lifetime may also induce epigenetic changes such as mutations, ageing, microbial exposure, smoking, stress, pollution and diabetes (Loos & Van Dyke, 2020). For example, smoking may alter the gene expression related to bone homeostasis, tissue repair and immune response thus activating the genetic predisposition to early-onset periodontitis (Freitag-Wolf et al., 2019). Several genes and single nucleotide polymorphisms (SNPs) have been found to be associated with periodontitis (Nibali et al., 2017). In this regard, the vitamin D receptor (VDR), Fc gamma receptor IIA (Fc-γRIIA) and Interleukin 10 (IL10) genes have gathered the most evidence in previous studies, however, for different gene variants/ SNPs and/or in different populations (Chapple et al., 2017; Nibali et al., 2017; Loos & Van Dyke, 2020). Thus, more research is still required before causality between specific genetic factors and periodontitis can be concluded (Kinane et al., 2017; Nibali et al., 2017; Loos & Van Dyke, 2020).

1.3 PATHOGENESIS OF PERIODONTITIS

1.3.1 PERIODONTAL INFLAMMATION WITH DYSBIOSIS TRANSFORMS GINGIVITIS TO PERIODONTITIS

The initiation and development of periodontitis is a complex interplay between polymicrobial biofilm (dental and subgingival plaque) and host inflammation and immune response (Colombo & Tanner, 2019; Hajishengallis et al., 2020; Van Dyke et al., 2020). Accumulation of bacterial plaque around the tooth at and below the gingival margin leads to gingival inflammation and gingivitis (Löe et al., 1965). The inflammation process in gingivitis is restricted

to gingiva and no bone loss occurs, unlike in periodontitis (Kinane et al., 2017). Gingivitis is a reversible state of disease in which the local inflammation and the occurred tissue changes can be reverted if dental biofilm is removed or disrupted (Armitage, 2004). Thus, management of gingivitis plays an important role in primary and secondary prevention of periodontitis (Murakami et al., 2018).

Gingivitis is considered a major risk factor for periodontitis and a precursor of periodontitis (Lang et al., 2009). It is still not perfectly clear when and why gingivitis transforms from a local and restricted inflammatory response to a progressing and destructive periodontitis (Kurgan & Kantarci, 2018, Van Dyke et al., 2020). But it seems to have to do with the disruption of the bi-directional balance (homeostasis) between the host's inflammatory response and the subgingival biofilm (Curtis et al., 2020). Changes in the diversity and proportions of the bacterial species in the subgingival biofilm has been associated with initiation of periodontitis, yet clear evidence for specific bacteria as the root cause for initiation of periodontitis is still lacking (Lamont et al., 2018; Bartold et al., 2019). But it seems that at the later stages of periodontitis specific periodontal pathogens may start to play a role in dysregulation of the host defense and increasing attachment loss and disease progression (Van Dyke et al. 2020). Whether dysbiosis is the consequence or the initiating factor for periodontitis is still to be confirmed (Van dyke et al., 2020). In that regard, it seems that both the dysbiosis and pocket formation together with chronic, excessive, and uncontrolled host inflammation and immune response may be able to reinforce each other in a feed-forward cycle, which transforms gingivitis to periodontitis (Marsh, 1990; Hajishengallis et al., 2012; Lamont et al., 2018; Hajishengallis et al., 2020; Hajishengallis & Lamont, 2021). Periodontal inflammation and related tissue destruction, which is largely mediated by neutrophils, provides these dysbiotic communities nutrients, such as degraded collagen that supports the progression of the lesion (Hajishengallis & Korostoff, 2017).

1.3.2 INNATE AND ADAPTIVE IMMUNITY NETWORK IN THE PATHOGENESIS OF PERIODONTITIS

Overall, the immunological network in the gingival tissues is rich and diverse involving elements of both innate and adaptive immunity (see Figure 2) (Moutsopoulos & Konkel, 2018; Hajishengallis et al., 2020). The system is based on complex interactions between neutrophils, T cells, and antigen-presenting cells, including B cells, macrophages, and dendritic cells and humoral system, including the complement system (Hajishengallis et al., 2020). The innate immunity is characterized by non-specific responses against invading pathogens, while the adaptive immunity involves specialized immune cells (B cells and T cells) and antibodies that utilize recognition, memory and binding to eliminate invading pathogens (Hajishengallis et al., 2020). When the tissue homeostasis is disrupted by tissue destructive

inflammation and a shift from the symbiotic, commensal bacterial communities towards dysbiotic ones, this system of innate and adaptive immunity can mobilize the pathological activation of osteoclasts leading to alveolar bone resorption (Hajishengallis & Lamont, 2021). It is known that bone forming osteoblasts, as well as, activated T cells, B cells, and neutrophils can express membrane-bound receptor activator of nuclear factor-κB ligand (RANKL), which binds to its receptor RANK on osteoclast precursors and initiates osteoclast differentiation and activation (Hajishengallis & Korostoff, 2017). Here, the soluble decoy receptor osteoprotegerin is able to block the RANKL-RANK function and regulate the osteoclastogenesis (Hajishengallis & Korostoff, 2017).

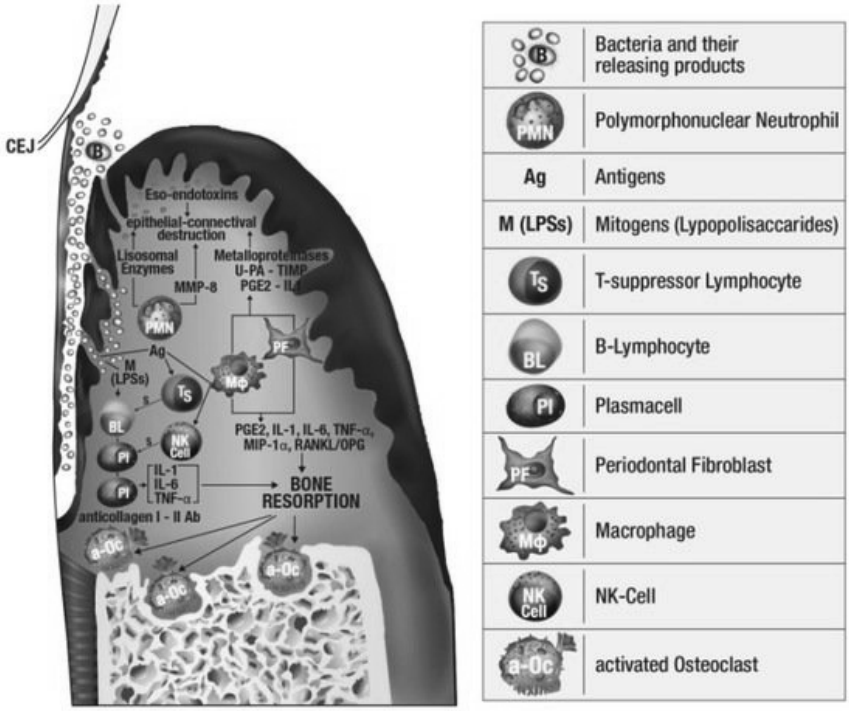

Figure 2 The rich and diverse immunological network in the periodontal tissues releasing several products during immune reaction against microbial challenge invading from the periodontal pocket. Reproduced from Cafiero et al (2021) under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license https://creativecommons.org/licenses/by/4.0/.

Even in a healthy periodontium, there is always an inflammatory cell infiltrate in the gingival tissues, which is part of the normal physiological immune surveillance process (Lang and Bartold, 2018). Neutrophils are the dominating cell type in a healthy periodontium and at the initial stages of gingivitis (Page & Schroeder, 1976). They are the first cells that are recruited from the circulation to the periodontium in response to periodontal

inflammation (Hajishengallis et al., 2020). Neutrophils are a major anti-microbial phagocyte that also has a large arsenal of other anti-microbial resources and compounds, such as neutrophil elastase, and matrix metalloproteinases, against the bacterial challenge (Uriarte & Hajishengallis, 2022). Neutrophils also have immunomodulatory roles that link innate and adaptive immunity cells and their responses, which has a major contribution to the maintenance of periodontal tissue homeostasis (Uriarte & Hajishengallis, 2022). Their role in the pathogenesis is discussed more in detail in the next section. In a healthy periodontium, neutrophils are accompanied by T cells and in lesser amounts macrophages, B cells and plasma cells (Page & Schroeder, 1976).

The main T cell type in periodontal health is CD4-positive T helper cells, but there are also cytotoxic CD8-positive T cells and other T cells in lesser amounts (Hajishengallis & Korostoff, 2017). T cells are part of the local immune defense and tissue homeostasis (Hajishengallis & Korostoff, 2017). Naive CD4-positive T helper cells can be activated by antigen-presenting cells, including dendritic cells, to differentiate into different effector T helper (Th) cell types, namely Th1, Th2, Th17 and regulatory T (Treg) cells, that have distinct functions and cytokine expression characteristics (Hajishengallis & Korostoff, 2017). For example, Th17 can regulate neutrophil recruitment, while Tregs can downregulate effector functions of Th17 and other T helper cells (Hajishengallis & Korostoff, 2017). Macrophages are anti-microbial phagocytes but can also increase expression of proinflammatory cytokines, costimulating molecules, such as CD86, which is required for T cell activation, and antimicrobial molecules, such as reactive oxygen species (ROS) (Hajishengallis & Korostoff, 2017). Macrophages are also important in the resolution of inflammation with their ability, for example, to regulate levels of proinflammatory and anti-inflammatory mediators in order to end the neutrophil recruitment and to clean up apoptotic neutrophils by efferocytosis (Hajishengallis & Korostoff, 2017).

B cells function in immune and inflammatory responses by secreting cytokines but also function as antigen-presenting cells (Hajishengallis & Korostoff, 2017). Microbial antigens can activate mature or naive B cells into differentiating into antibody-secreting plasma cells or memory cells against the activating microbial antigen (Hajishengallis & Korostoff, 2017). In most cases, this requires a microbial antigen interaction with the B-cell antigen receptor (BCR) together with a secondary stimulation from T cells (T-dependent antigens) (Hajishengallis & Korostoff, 2017). There are also (T-independent) antigens, for example, bacterial polysaccharides, that are able to activate B cells without the help of T cells by receiving additional signals from toll-like receptors (TLRs) or by cross-linking of multiple BCRs (Hajishengallis & Korostoff, 2017).

As the severity of the inflammation process in the periodontium progresses towards periodontitis the composition and complexity of the cell infiltrate changes in the periodontium. Then, although the number of neutrophils

increases, the increase in the number of macrophages and T-cells is much larger, and eventually B cells and plasma cells begin to prevail in periodontitis (Page & Schroeder, 1976; Thorbert-Mros et al., 2015). The complement system is an important mediator in neutrophil-mediated inflammation regarding neutrophil recruitment into tissues and effector functions, but it also modulates the differentiation of T cells and B cells (Hajishengallis et al., 2020; Uriarte & Hajishengallis., 2022). There seems to be significantly higher complement activation product levels in the gingival tissue and the gingival crevicular fluid of periodontitis patients compared with healthy subjects (Hajishengallis et al., 2020).

1.3.3 MANY ROLES OF NEUTROPHILS IN THE PATHOGENESIS OF PERIODONTITIS

Neutrophils are the most abundant leukocyte type (≥ 95 %) in the gingival crevice (Delima & Van Dyke, 2003). Neutrophils are constantly entering from the circulation into the gingival tissues from where they migrate to the junctional epithelium and finally into the gingival crevice/sulcus (Schroeder & Listgarten, 1997). The process is coordinated and regulated by chemokines, such as complement activator products (e.g., the anaphylatoxin C5a) and chemoattractive bacterial peptides, and adhesion molecules whose increasing concentration (chemokine gradient) directs the migration of neutrophils towards gingival crevice (Kolaczkowska & Kubes, 2013; Hajishengallis et al., 2020). A well-functioning neutrophil recruitment is an essential part of a healthy gingiva (Hajishengallis & Hajishengallis., 2014). Several genetic neutrophil defects and conditions disrupting the neutrophil homeostasis, such as neutropenia and leukocyte adhesion deficiencies, have been shown to increase the susceptibility to and severity of periodontitis (Hajishengallis & Hajishengallis., 2014). Chronic periodontitis is associated with decreased neutrophil chemotactic accuracy increasing the number of neutrophils recruited and risk of increased tissue destruction (Roberts et al, 2015).

Neutrophils in the gingival tissues play an important role in periodontal immunity as well as in periodontal health and homeostasis (Scott & Krauss, 2012; Moutsopoulos & Konkel, 2018; Hajishengallis et al., 2020; Uriarte & Hajishengallis, 2022). They form a first line of defense, an antimicrobial wall between the junctional epithelium and the microbial challenge (subgingival biofilm) (Scott & Krauss, 2012). They are anti-microbial phagocytes (predominant ones in bacterial infections) that have a robust secretory structure of oxygen-dependent and oxygen-independent (lytic and proteolytic enzymes) as well as neutrophil extracellular bacterial traps (NETs) for protecting the host from bacterial challenge (Scott & Krauss, 2012; Hajishengallis & Korostoff, 2017; Moutsopoulos & Konkel, 2018). Neutrophils sense the microbial structures and target microbial organisms by using pattern recognition receptors (PRRs), most importantly toll-like receptors (TLRs) (Uriarte & Hajishengallis, 2022). Activated neutrophils in the periodontium

can contribute to bone demineralization and matrix degradation by expressing an important osteoclastogenic cytokine RANKL and releasing matrix metalloproteinases, such as neutrophil collagenase (matrix metalloproteinase-8), respectively (Lee et al., 1995; Sorsa et al., 2006; Hajishengallis & Korostoff, 2017).

In addition to killing microbial challenge with granule-secreted molecules and enzymes, neutrophils seem to have proinflammatory, anti-inflammatory and immunoregulatory roles, and interact with resident cells in the periodontium and other leukocytes (Uriarte & Hajishengallis, 2022). They can promote the recruitment of CD4+ T helper cells that release interleukin-17 (Th17 cells) to sites of infection or inflammation as well as promote the survival, proliferation, and development of B cells into plasma cells that secrete antibodies (Hajishengallis & Korostoff, 2017). Neutrophils can also downregulate the host immune response by directly suppressing T cell activation and responses as well as indirectly inhibiting the antigen presenting function of dendritic cells (Hajishengallis & Korostoff, 2017). Furthermore, neutrophils can inhibit activation and promote anti-inflammatory phenotype in many innate immune cells, such as macrophages and natural killer cells suggesting a role in suppressing unnecessarily high and potentially harmful T cell activation in periodontitis (Hajishengallis & Korostoff, 2017).

While neutrophils maintain periodontal homeostasis in health, their defense mechanisms seem to be inadequate to control a dysbiotic microbial community even if they are constantly recruited to the periodontium where they exert their immune and inflammatory responses (Uriarte & Hajishengallis, 2022). Several inflammatory mediators such as cytokines, chemokines and microbial derived pathogen associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), can prime neutrophils that can make them to overreact against the microbiota at the gingival crevice (Uriarte & Hajishengallis, 2022). Neutrophil hyperactivity and hyperreactivity as well as inaccurate chemotaxis leading to increased recruitment of neutrophils have been associated with chronic periodontitis by increasing the release of inflammatory and cytotoxic mediators and enzymes causing enhanced collateral damage to the tissues (Hajishengallis & Korostoff, 2017). Moreover, there are age-related alterations in the recruited neutrophil function, which cause unfocused or inaccurate chemotaxis as well as elevated release of tissue destructive proteases in elderly subjects (Sapey et al., 2014). These inflammatory and hyperactivity alterations in neutrophil functions seem to persist despite successful periodontal treatment (Hajishengallis & Korostoff, 2017).

1.4 MATRIX METALLOPROTEINASES (MMPS)

1.4.1 INTRODUCTION TO MMPS

Matrix metalloproteinases (MMPs) are an important family of zinc-dependent endopeptidases capable of degrading various proteins in the extracellular matrix (ECM), such as collagens, gelatins, and elastins, but also several non-ECM proteins, such as cytokines, chemokines, growth factors, and cell surface membrane proteins (Sorsa et al., 2006; Iyer et al., 2012; Nissinen & Kähäri, 2014; Cui et al., 2017; Bassiouni et al., 2021). As of the 28 members of MMP family in vertebrates, at least 23 members have been found in the human genome (Cui et al., 2017). MMPS are usually classified into six subgroups that are based on their substrate specificity and structural domains: collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2, and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7, and -26), membrane-type MMPs (MT-MMPs) (MMP-14, -15, -16, -17, -24, -25), and other MMPs (MMP-12, -19, -20, -21, -22, -23, -27, -28) (Cui et al., 2017).

The members of MMP family have a similar core structure, which typically consists of four distinct domains: N-terminal pro-peptide domain, catalytic domain, signal domain (a linker peptide/ hinge region), and C-terminal hemopexin-like domain (Sorsa et al., 2006). It is worth noting that MMPs are expressed as inactive proenzymes i.e., zymogens and turned into proMMPs by removing the signal peptide during the translation (Nagase et al., 2006). The interaction between the catalytic domain (Zn^{2+}) and the propeptide domain (cysteine switch) is essential for keeping proMMPs in inactive state as it prevents a water molecule binding to the zinc atom and resulting catalysis and proMMP activation (Nagase et al., 2006). Catalytically competent active MMP forms are generated by processing these latent zymogens and proMMPs by proteolytic enzymes, such as serine and cysteine proteinases as well as other MMPs that cleave the cysteine switch and detach the pro-peptide domain (Sorsa et al., 2006; Nagase et al., 2006).

Multiple tissues and cells are able to produce and secrete MMPs such as variety of connective tissue, and proinflammatory cells including neutrophils, macrophages, lymphocytes, fibroblasts, endothelial cells, and osteoblasts (Verma & Hansch, 2007; Cui et al., 2017). MMPs are involved in a large number of physiological and pathological processes, such as tissue remodeling, wound healing, cell apoptosis, periodontal disease, osteoarthritis, and cancer (Verma & Hansch, 2007; Cui et al., 2017; Bassiouni et al., 2021). MMPs and their proteolytic activity are regulated in normal physiological state by controlling the level of mRNA expression, activation of latent zymogen and proMMPs, and inactivation of active MMPs by endogenous tissue inhibitors of MMPs (TIMPs) (Cui et al., 2017). The balance between active and inactive MMPs is a complex process regulated by gene expression, activation of inactive proenzymes and endogenous inhibition, but also cell-ECM and cell-cell interactions and bioactive products of ECM proteins may play additional

role in this regulation (Gaffney et al., 2015; Cui et al., 2017). In harmful pathological conditions this balance (tissue homeostasis) is disrupted leading to disease (Verma & Hansch, 2007; Cui et al., 2017). That makes MMPs and their regulators potential therapeutic targets and biomarker candidates for many pathological conditions (Verma & Hansch, 2007; Cui et al., 2017).

1.4.2 MMP-8, ITS ACTIVATION, AND ITS ROLE IN PERIODONTAL DISEASES

Collagenase-2 (MMP-8), also known as neutrophil collagenase, was at first isolated and sequenced from neutrophils (Sorsa et al., 1985; Sorsa, 1987; Hasty et al., 1990). MMP-8 is produced during the maturation of neutrophils in the bone marrow and the latent enzyme is stored in the subcellular specific granules in glycosylated form to be released by a selective degranulation when an appropriate triggering stimulus occurs (Sorsa et al., 2006; Borregaard et al. 2007). In addition to neutrophils, several other cells are also able to express MMP-8, for example, synovial and gingival fibroblasts, macrophages/monocytes, plasma cells, epithelial cells/keratinocytes, odontoblasts, articular chondrocytes, and oral cancer cells (Sorsa et al., 2006). For example, impaired neutrophil function in juvenile periodontitis allows colonization of periodontopathogenic bacteria that may be able to induce the resident periodontal cells to produce increased amounts of collagenase (Suomalainen et al., 1991). However, the majority of different MMP-8 species in GCF, saliva and gingival tissues is derived from neutrophils (Gangbar et al., 1990; Uitto et al., 1990; Kiili et al., 2002).

It is known that not only other MMPs but also pro-inflammatory mediators, such as tumor necrosis factor (TNF)-alpha and IL-1, and bacterial virulence factors, such as LPS, as well as, other chemical stimulus, such as reactive oxygen species (ROS), are able to promote neutrophil degranulation to release the latent MMP-8 from the granules to be further activated, which initiates periodontal tissue destruction cascades (Cui et al., 2017, Sorsa et al., 2006; Sorsa et al., 2016). Likewise, degranulation of myeloperoxidase (MPO) from neutrophils can catalyze the hypochlorous acid (HOCl) production from hydrogen peroxide (H_2O_2) that is a potent activator of extracellular MMP-8 (Sorsa et al., 2006). Furthermore, periodontopathogenic *P. gingivalis* and *T. denticola* with (the chymotrypsin-like proteases) gingipains and dentilisin, respectively, have been shown to be able to trigger the secretion and activation of the latent MMP-8 (Ding et al., 1996; Imamura et al., 2003; Nieminen et al., 2018; Gürsoy et al., 2018; Petain et al., 2021). Interestingly, dentilisin is able to activate proMMP-8 in only 20 minutes (Nieminen et al., 2018; Petain et al., 2021).

The prospective study by Lee et al (1995) was one of the first demonstrating a direct role of collagenolytic activity by active MMP-8 (aMMP-8) in the progression of periodontal connective tissue breakdown and periodontitis. On the other hand, a recent longitudinal study showed that aMMP-8 can be

regarded as a significant predictor for peri-implantitis (Guarnieri et al., 2022). Furthermore, several other studies have shown elevated active MMP-8 levels in periodontitis in adults and adolescents, but also that they are reduced after periodontal treatment (Mancini et al., 1999; Romanelli et al., 1999; Kiili et al., 2002; Mäntylä et al., 2003; Mäntylä et al., 2006; Sorsa et al., 2010; Leppilahti et al., 2011; Leppilahti et al., 2014a; Leppilahti et al., 2014b; Izadi Borujeni et al., 2015; Leppilahti et al., 2015; Heikkinen et al., 2016a; Heikkinen et al., 2016b; Johnson et al., 2016; Heikkinen et al., 2017a; Heikkinen et al., 2017b; Lorenz et al., 2017; Alassiri et al., 2018; Deumer et al., 2019; Grigoriadis et al., 2019; Schmalz et al., 2019; Taylor et al., 2019; Sorsa et al., 2020; Keles Yucel et al., 2020; Lähteenmäki et al., 2020; Raivisto et al., 2020a; Raivisto et al., 2020b; Raivisto. 2020c; Deng et al., 2021a; Hernández et al., 2021; Öztürk et al., 2021; Deng et al., 2022; Heikkinen et al., 2022; Umeizudike et al., 2022). The active MMP-8 seems also diagnostically more useful than total MMP-8 (the latent and active species), as two recent studies found that there was no significant difference in the gingival tissue levels of total MMP-8 between periodontitis, peri-implantitis, and periodontally healthy control patients (Karatas et al., 2020; Petain et al., 2021). Furthermore, it has been recently shown by using Western blot analysis that active forms of MMP-8 exist in the saliva of periodontitis patients but not in the saliva of gingivitis patients during the gingival inflammation and its resolution (Gürsoy et al., 2018; Silbereisen et al., 2020). Periodontal soft and calcified tissues are mostly comprised of type I and III collagens (Romanos & Bernimoulin, 1990). In that regard, host modulation therapy of collagenolytic activity with systemic administration of non-antibiotic tetracyclines, chemically modified non-antimicrobial tetracycline derivatives, and subantimicrobial-dose doxycycline (SDD) have been shown to reduce collagenase activity in GCF during periodontitis, particularly MMP-8 activity that is the most dominant collagenase in GCF, and correlating with patient's clinical periodontal improvements (Golub et al., 1995; Reinhardt et al., 2010; Sorsa et al., 2011; Golub et al., 2016; Emingil et al., 2019; Golub & Lee, 2020). Similarly, chlorhexidine (CHX) with the antiseptic and gingival inflammation reducing properties has been shown to reduce collagenolytic activity of MMP-8 (Gendron et al., 1999). Furthermore, previous studies suggest that also bisphosphonates, kaempferol, blueberries, lingonberries, tenoxicam could be beneficial for decreasing MMP-8 levels (Teronen et al., 1997; Teronen et al., 1999; Ozgören et al., 2014; Ben Lagha et al., 2015; Balli et al., 2016; Lähteenmäki et al., 2022a).

1.4.3 AMMP-8 DETECTION BASED ON ANTIBODIES AND IMMUNOASSAYS

Several antibody-based detection methods have been developed to measure aMMP-8 levels. Traditional standard laboratory methods, including time-resolved immunofluorometric assay (IFMA) and enzyme-linked immunosorbent assay (ELISA), can detect MMP-8 levels in oral fluids. aMMP-

8 IFMA analysis can be used for aMMP-8 detection when based on aMMP-8 specific monoclonal antibodies 8708 and 8706 (Oy Medix Biochemica Ab, Espoo, Finland). Many commercial ELISA kits (Quantikine Human Total MMP-8 Immunoassay R&D Systems™) detect both latent and active forms of MMP-8, i.e., total MMP-8. aMMP-8 specific detection is possible with DentoELISA kits (Dentognostics GmbH, Jena, Germany) utilizing the same aMMP-8 IFMA antibodies 8708 and 8706. In that regard, comparisons between aMMP-8 IFMA and commercial ELISA has shown aMMP-8 IFMA to be more accurate in periodontal disease diagnostics, while, with the same aMMP-8 antibodies, DentoELISA correlates strongly with aMMP-8 IFMA (correlation = 0.881-0.95, $p \leq 0.01$) (Sorsa et al., 2010; Leppilahti et al., 2014b; Nieminen et al., 2015).

Recently, aMMP-8 Point-of-Care technologies (POCT) have been studied extensively for rapid aMMP-8 chairside/bedside detection for periodontitis diagnostics demonstrating an association with periodontal clinical parameters, the community periodontal index of treatment needs (CPITN) index and disease progression (Nwhator et al., 2014; Izadi Borujeni et al., 2015; Heikkinen et al., 2016a; Johnson et al., 2016; Heikkinen et al., 2017b; Lorenz et al., 2017; Alassiri et al., 2018; Räisänen et al., 2018; Grigoriadis et al., 2019; Schmalz et al., 2019; Keskin et al., 2020; Lähteenmäki et al., 2020; Sorsa et al., 2020; Deng et al., 2021a; Öztürk et al., 2021; Deng et al., 2022; Umeizudike et al., 2022). Furthermore, there have been found a significant association between aMMP-8 POCT and staging and grading of periodontitis (the 2017 classification system of periodontitis) (Tonetti et al., 2018; Sorsa et al., 2020). In that regard, aMMP-8 has been suggested to be implemented as the biomarker for the classification system of periodontitis (Sorsa et al., 2020; Sorsa et al., 2022). Finally, previous studies have shown that aMMP-8 POCT has a good agreement with aMMP-8 IFMA and DentoELISA, which they all utilize the same two antibodies (Lorenz et al., 2017; Alassiri et al., 2018).

There has also been introduced a new MMP-8 detection method based on specific antibodies and surface acoustic wave (SAW) biosensor technology (Taylor et al., 2019). The device seems to correlate more with ELISA (R&D Systems™) (correlation = 0.681, $p < 0.001$) than aMMP-8 IFMA (correlation = 0.354, $p < 0.001$), which suggests that SAW biosensor detects mostly total MMP-8 rather than aMMP-8 (Taylor et al., 2019; Umeizudike et al., 2022). Finally, other independent catalytic activity aMMP-8 assays have been studied, as well, but their correlation with other methods is still to be studied (Lee et al., 1995; Mancini et al., 1999; Romanelli et al., 1999; Kiili et al., 2002; Gul et al., 2016; Mc Crudden et al., 2017).

2 AIMS AND HYPOTHESIS OF THE STUDY

This study aims at describing the main risk factors of subclinical/initial periodontitis in Finnish adolescents aged 15-17 years, and how the factors could be utilized in the identification and prevention of subclinical/initial periodontitis.

The specific aims of this study are:

Study I
To investigate how different patient-related risk factors may be associated with the odds of developing subclinical/initial periodontitis in adolescents.

Study II
To compare the effectiveness of the aMMP-8 PoC mouthrinse test to the traditionally used whole mouth BOP (cutoff of 20% for positives) test for detection of subclinical/initial periodontitis in adolescents.

Study III
To examine the relative impact of different local and modifying factors on whole-mouth BOP levels in adolescents.

Study IV
To compare the diagnostic utility of salivary and mouthrinse analytics of aMMP-8 for discriminating periodontal health and disease.

3 MATERIALS AND METHODS

3.1 STUDY POPULATION

This dissertation study examined initial periodontitis and its risk factors in two birth cohorts of 15-17-year-old adolescents living in the city of Kotka, Finland, in 2004-2005 (born in 1989–1990) and in 2014–2015 (born in 1999–2000). The sample of 2004–2005 consisted of 501 adolescents (258 boys and 243 girls) after 44 adolescents out of original 545 subjects had refused to take part to an examination for unknown reasons. Similarly, the sample of 2014–2015 consisted of 47 adolescents (30 boys and 17 girls) that had accepted the invitation to an examination. For reasons that remained unknown, 72 adolescents out of the whole birth cohort (120 adolescents) had refused to take part. Furthermore, one participant was excluded from the sample of 2014–2015 due to a discrepant gender check, after which there were total of 47 participants for the analysis. All participants filled a structured questionnaire including information about their general health and diseases and health behavior such as smoking status and its duration in pack years [cigarettes smoked per 20 X duration in years] as well as toothbrushing frequency. The majority of the participants were healthy and only in the sample of 2004–2005 there were 18 adolescents that had allergies, 12 adolescents that had respiratory disease and 10 adolescents that had skin diseases. The mixed dentition stage was finished for all the participants, and none of the participants was under orthodontic treatment. This study was conducted according to the principles of the Declaration of Helsinki. The study protocol had an approval from the Ethical Committees of the Kymenlaakso Central Hospital and the Helsinki University Central Hospital (Diary number 260/13/03/00/13). The age of consent is 15 years in Finland. Therefore, all participants were eligible to provide a written informed consent, and parental consent was not required by the institutional review board.

3.2 CLINICAL EXAMINATION (STUDIES I-IV)

Clinical examination for recording the oral health status of all participants in the 2004–2005 and 2014–2015 samples was conducted by one calibrated and a specially trained dentist, a periodontist (AMH), who was a priori unaware of the smoking status of the adolescents. Visible plaque index (VPI%) and root calculus (RC%) percentages were recorded using the World Health Organization (WHO) index teeth (dd. 16, 11, 24, 36, 41, 44). Bleeding on probing percentage (BOP%) and probing depth (PD) were examined from all the teeth at four and six probing sites, respectively. All at least 4 mm deep

periodontal pockets were recorded. An oral radiologist assessed bilateral bitewing radiographs for interproximal bone/attachment loss (AL) of the premolars and molars. AL was recorded if the distance between the crestal bone margin and the cemento-enamel junction (CEJ) was more than 2 mm. AL less than 2 mm was considered normal. As has been published before, in this sample, 14.6 % of adolescents had AL ≥ 2 mm (Heikkinen, 2011).

3.3 SUBGINGIVAL POOLED PLAQUE SAMPLES AND ANALYSIS (STUDIES I AND III)

Subgingival pooled plaque samples were collected before the clinical examination from the teeth that had at least 3 mm probing depth as described in Heikkinen (2011). The tooth was dried and isolated with cotton rolls before using a sterile paper point to collect the subgingival sample. For practical reasons and based on pre-study power calculation in the sample of 2004–2005, the samples were collected from 264 adolescents (31.4 % were smokers, 62.9 % non-smokers and 5.7 % former smokers) (Heikkinen, 2011). The plaque samples were placed in 100 µl of sterile water and stored at -75°C after which polymerase chain reaction (PCR) analysis was used to detect the putative periodontopathogens *Aggregatibacter actinomycetemcomitans* (*A.a.*), *Porphyromonas gingivalis* (*P.g.*), *Prevotella intermedia* (*P.i.*), *Prevotella nigrescens* (*P.n.*), *Tannerella forsythia* (*T.f.*), and *Treponema denticola* (*T.d.*) with specific primers described in Wahlfors et al. (1995) and Meurman et al. (1997). In short, as Heikkinen (2011) describes, a one minute was used to centrifuge the thawed samples at 2100 x g, and 5 µl aliquots of the supernatants were added to the PCR reaction mixture so the final volume was 50 µl. Furthermore, the enzyme used for the PCR amplification was Dynazyme II Hot Start DNA Polymerase (Finnzymes, Espoo, Finland) in the GeneAmp® PCR System (Perkin-Elmer Corporation, Norwalk, CT, USA) (Heikkinen, 2011).

3.4 SALIVARY SAMPLES AND ANALYSIS (STUDIES I, III AND IV)

Stimulated salivary samples (about 5 ml) were collected between 8 a.m. and 3 p.m. after the clinical examination. The participants first rinsed their mouths with water and then chewed a small piece of paraffin wax. The saliva secreted within the first 30 s was swallowed and saliva was collected for the next 5 min. The stimulated saliva samples were immediately centrifuged at 1000 x g for 5 minutes and then the collection of the saliva samples and the supernatants were frozen at -20°C and kept at -80°C until assayed for the enzyme analysis as described in Heikkinen (2011), Uitto et al (1990), Ingman et al (1993) (PMN elastase), Helenius et al (2005) (IgA, IgG, IgM), and Umeizudike et al (2022)

(aMMP-8 IFMA). They were later assessed for aMMP-8 (μg/l) (N = 499), PMN elastase activity (ΔOD405/h) (N = 497), immunoglobulin A (IgA; μg/l) (N = 262), immunoglobulin G (IgG; μg/l) (N = 262), immunoglobulin M (IgM; μg/l) (N = 262), total protein (μg/l) (N = 262), albumin (μg/l) (N = 262).

Salivary aMMP-8 levels were analyzed by time-resolved immunofluorometric assay (IFMA) utilizing the monoclonal MMP-8-specific antibodies 8708 (a catching antibody) and 8706 (a tracer antibody), where the specificity of the monoclonal antibodies against MMP-8 corresponds to that of polyclonal MMP-8 (Hanemaaijer et al., 1997). In this method, the assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mm CaCl2, 50 μM ZnCl2, 0.5 % bovine serum albumin, 0.05 % sodium azide, and 20 mg/l diethylenetriaminepentaacetic acid. The salivary samples were diluted with assay buffer and pipetted into the 96 wells microtitration plates. After one hour of incubation, the wells were washed by washing buffer and then the wells were incubated with the tracer antibody labelled with Europium for one hour after which an enhancement solution was added. The fluorescence was measured after five minutes using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland), nowadays an EnVision 2105 multimode plate reader (PerkinElmer Finland, ex. Wallac, Turku, Finland). The detection limit for the assay is 0.08 ng/ml (Hanemaaijer et al., 1997, Umeizudike et al., 2022).

PMN elastase activity was measured using the difference in the optical density unit (OD) values before and after one hour incubation detected by spectrophotometer at 405 nm as described by Nieminen et al (1993). Briefly, in this assay N-succinyl-Ala-Ala-Val p-nitroanilide (SAAVA) was used as substrate that PMN elastase in the saliva samples was able to cleave so that p-nitroanilide was released which simultaneously changes the color of the solution to yellow. The optical intensity of the yellow color was measured at a wavelength of 405 nm correlating with the activity of PMN elastase as a function of time.

Immunoglobulins IgA, IgG and IgM were analyzed by a modified enzyme-linked immunosorbent assay (ELISA) described previously in Lehtonen et al (1984). In short, the antibodies in the catching layer were rabbit anti-human IgG (or IgA or IgM; Dako A/S, Glostrup, Denmark), and the secondary antibodies were peroxidase-conjugated rabbit antihuman IgG (or IgA or IgM, Dako A/S). The control curve was defined from the human serum standard with known amounts of IgG, IgA and IgM (Behringwerke AG, Marburg, Germany) and was further used to calculate the immunoglobulin concentrations.

Total protein was measured by the colorimetric Lowry method (Lowry et al., 1951) as described and performed by Helenius et al (2005). Albumin was analyzed according to Webster et al (1977). All determinations were carried out as duplicates immediately after thawing, and standards and controls were included in all.

3.5 AMMP-8 POINT-OF-CARE MOUTHRINSE TEST (STUDIES II AND IV)

The aMMP-8 point-of-care mouthrinse test (PerioSafe, Dentognostics GmbH, Jena, Germany) is a lateral-flow immunoassay test that has been developed by Medix Biochemica Ltd (Espoo, Finland) and Dentognostics GmbH (Jena, Germany). The test was performed in this study by a periodontist (AMH) in accordance with the manufacturer's instructions. All the needed equipment to perform the aMMP-8 mouthrinse test are included in the test kit. The mouthrinse collection consists of three phases: 1) patient prerinses with tap water for 30 seconds, 2) waits for 30 seconds, after which 3) patient rinses 30 seconds with the test liquid (aqua purificata). The mouth rinse is collected by pouring it into a small collection beaker from which three milliliters of mouthrinse are drawn into a syringe that is together with a filter used for placing three drops (maximum is four drops) on the test stick, which is inserted to the aMMP-8 lateral-flow immunoassay test system. The test result becomes visible (one or two lines) after five minutes based on the color change resulting from immunoreactions in the test stick. One blue line indicates a negative test result (no risk), while two blue lines indicate a positive test result (increased risk for periodontitis). Even a thin second line represents a positive test result. Thus, the aMMP-8 mouthrinse test resembles a pregnancy test. During data analyses the test positive results (thin and clear second lines) were combined as one group. Two blue lines (a positive test) indicates aMMP-8 levels ≥ 20 ng/ml (cut-off point for the aMMP-8 mouthrinse test).

The aMMP-8 chairside test is based on the immunochromatography principle involving two highly MMP-8 specific monoclonal antibodies 8708 (a catching antibody) and 8706 (a tracer antibody), where the specificity of the monoclonal antibodies against MMP-8 corresponds to that of polyclonal MMP-8 (Hanemaaijer et al., 1997). The test works on the basis of a lateral-flow sandwich immunoassay. The three drops of mouthrinse in the system flows towards the two antibodies attached to the latex particles on the test stick. If there is any aMMP-8 in the mouthrinse sample, it binds to the first antibody and the particles flow along the test draw to the other antibody, which acts as the detecting label (Mäntylä, 2006).

3.6 STUDY DESIGNS AND STATISTICAL ANALYSIS

Study I

Study I investigated different potential risk factors for initial periodontitis in adolescents in the population based on a birth cohort (born in 1989–1990) examined in 2004–2005 (N = 501; see also Section 3.1). The factors studied included the number of sites with the presence of ≥ 4mm probing depth (PD#), AL, BOP%, VPI% and RC%, A.a., P.g., P.i., P.n., T.f. and T.d., salivary aMMP-8 (by IFMA) and total protein, albumin, IgA, IgG, IgM and PMN elastase

activity. The study used a subset of the original population because bacteria and salivary samples were not available for all adolescents (see Sections 3.3 and 3.4 as well as Figure 3). The final size of the subset was 252 adolescents (141 boys and 111 girls).

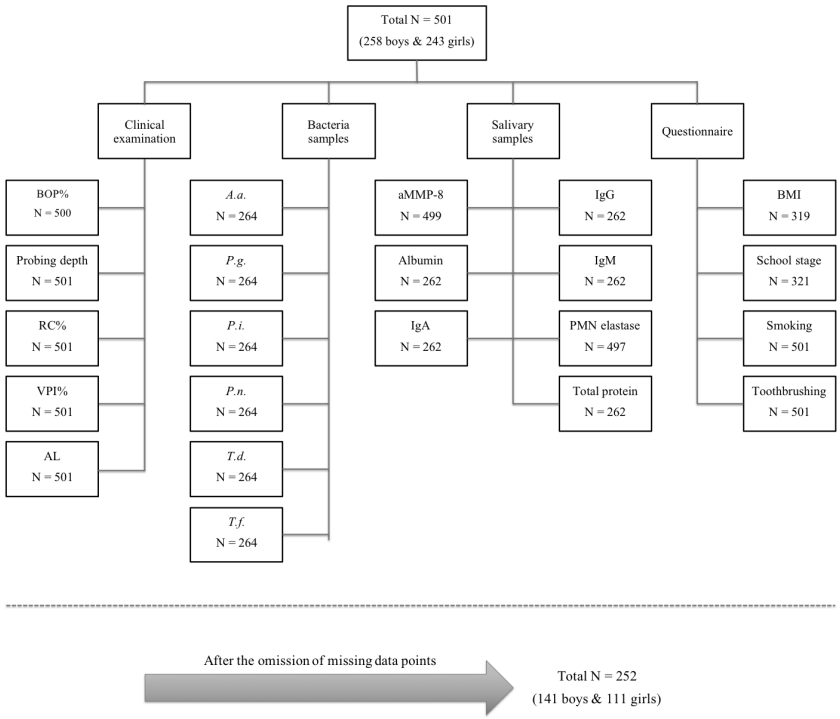

Figure 3 Study I population. A flow chart as presented in Heikkinen et al (2019) showing the number of adolescents with data recorded by a clinical examination, bacteria and salivary samples, and a questionnaire with a final sample size of 252 adolescents.

Study I used ordinal logistic regression analysis to investigate which factors were associated with the deterioration of periodontal health (no periodontal pockets) towards initial periodontitis (periodontal pocketing with groups of subjects in the presence of between 1–3, 4–7 and over 7 at least 4mm deep pockets). It can be approximated that as the number of sites with ≥ 4mm probing depth increases, similarly does the risk for initial periodontitis increase. Clearly an adolescent with two sites of ≥ 4mm probing depths is not the same as another adolescent with 20 sites of ≥ 4mm probing depths. The ordinal regression model in this study was built according to the purposeful variable selection process (Hosmer & Lemeshow, 2000). The prediction model included independent variables and interaction variables but was also controlled for confounders that correlate with both the dependent variable and

at least one independent variable in the regression model. Furthermore, patient characteristics (gender, education level, smoking, body mass index (BMI), toothbrushing, attachment loss, and probing depth) and their association with the ordinal groups of the periodontal pocketing was analyzed by one-way ANOVA, Fisher exact test, or Kruskal–Wallis H test. SPSS Statistics (Version 25.0, IBM Corp, Armonk, NY) was used in all analysis and the significance was set at $p < 0.05$.

Study II

Study II compared the aMMP-8 PoC mouthrinse test to the traditionally used BOP 20% (whole-mouth BOP% with a cutoff 20%) for the detection of initial periodontitis among 47 adolescents (born in 1999–2000) examined in 2014–2015. Patients were categorized into healthy patients (no deep periodontal pockets) and patients having at least one, at least two, at least three, and at least four sites with the presence of ≥ 4mm probing depth (different case definitions for initial periodontitis). Here the initial periodontitis (or the subclinical periodontitis) was defined as having low clinical and X-ray manifestations of deterioration of periodontal health, in other words, an early stage of the disease that corresponds to the stage I or even a subclinical state (before stage I) of periodontitis under the new 2017 classification of periodontal diseases (Tonetti et al., 2018; Tonetti et al., 2019).

Diagnostic accuracy was assessed using cross tabulations (2 x 2 contingency tables) for these four cases vs. healthy patients when aMMP-8 PoC mouthrinse test and BOP 20% were used. Finally, the classification performance of both the aMMP-8 test and BOP 20% was assessed by the receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) in each of the four cases. The AUC statistic was approximated by the trapezoid method with three reference points from the ROC curves. Microsoft® Excel® for Mac 2011 was used in the analysis.

Study III

Study III examined the relative impact of different local and modifying factors on whole-mouth BOP levels in adolescents aged 15-17. The study consisted of combination of the two birth cohorts of adolescents (N = 548) examined in 2004-2005 and 2014-2015. The final sample size was 544 adolescents after four adolescents were excluded from the analysis due to missing data. The strength of correlation was assessed by Spearman's correlation coefficient in univariable analysis between BOP levels and VPI%, RC%, pack years of smoking, toothbrushing, and the salivary aMMP-8 levels. Multiple linear regression analysis and standardized beta coefficients and t-values were used for assessing the relative impact of these variables on BOP levels. SPSS Statistics (Version 25.0, IBM Corp, Armonk, NY) was used in all analysis and the significance was set at $p < 0.05$.

Study IV

Study IV compared the diagnostic utility of salivary and mouthrinse analytics of aMMP-8 for discriminating periodontal health and disease among 47 adolescents (born in 1999–2000) examined in 2014–2015. Patients were categorized according to their periodontal pocket status into having at least two sites of ≥ 4mm periodontal pockets and having zero or one ≥ 4mm periodontal pockets. A box plot was constructed and salivary aMMP-8 levels measured by IFMA were plotted against the two categories of periodontal pocket status. The box plot was used for detecting potential false positive and negative values in salivary and mouthrinse aMMP-8 measurements.

4 RESULTS

4.1 RISK FACTORS FOR INITIAL PERIODONTITIS IN ADOLESCENTS (STUDY I)

Risk factors for the deterioration of periodontal health towards initial periodontitis and its extent in 252 Finnish adolescents were analyzed by ordinal logistic regression analysis (see Table 1). The odds of initial periodontitis for boys was over 5 times that of girls. Similarly pack-years of smoking were significantly associated with initial periodontitis. Interestingly, boys and girls with different education levels benefitted differently from their toothbrushing. That may indicate potential differences in toothbrushing habits (e.g., length, thoroughness) at various education levels among the adolescents in this study. Elevated BOP levels, particularly together with the increasing levels of root calculus, were associated with initial periodontitis both as an independent and also as an interaction variable. There was a significant association between aMMP-8 and initial periodontitis, and also between IgG and initial periodontitis. However, elastase levels were not associated with initial periodontitis. Finally, the odds of initial periodontitis was larger for adolescents with overweight and underweight compared with normal weight. The logistic regression model also included an independent variable for root calculus and confounding variables for education level, IgM, toothbrushing, *T.d.* and *T.f.*, but their association with initial periodontitis did not reach significance in the final prediction model. In univariable analysis, the association between VPI and initial periodontitis was significant, but VPI was not selected to the final prediction model in the variable selection process. Similarly, root calculus, *T.d.* and *T.f.* were significant in the univariable analysis, although not significant in the final multivariable prediction model.

4.2 THE AMMP-8 POC TEST ASSOCIATES WITH INITIAL PERIODONTITIS STRONGER THAN BLEEDING ON PROBING (STUDY II)

The aMMP-8 PoC mouthrinse test was compared with bleeding on probing (BOP 20% test with a cutoff 20% for positives) for their association with the four case definitions of initial periodontitis or subclinical periodontitis among 47 Finnish adolescents from the city of Kotka (see Table 2). The aMMP-8 PoC test had 2.8–5.3 times stronger association (odds ratio) with initial periodontitis than BOP 20% test. Overall, the aMMP-8 PoC test had better performance than BOP 20% test in classifying healthy adolescents and adolescents with initial periodontitis measured by AUC (19–30% bigger), the accuracy (25–29% bigger) and Matthews correlation coefficient (MCC) (55–

68% bigger). The sensitivity of the aMMP-8 PoC test for initial periodontitis was at least two times greater compared with BOP 20% test, the frequency of false negatives was 17–52% smaller for the aMMP-8 PoC test, while both tests had the same specificity of 100% and false positives of 0%. Finally, among the 47 adolescents in this study, there were 11 adolescents that had 6–32 at least 4mm deep periodontal pockets. It is worth noting that they all had a positive aMMP-8 PoC test, while only four of them had BOP > 20% and 8 of them had BOP > 10%.

Table 1. *Multivariable ordinal logistic regression analysis model of the risk factors of deterioration of periodontal health towards initial periodontitis by the purposeful variable selection process by Hosmer and Lemeshow (2000) (N = 252).*

Normal variables	**OR (95% CI)**	**Confounders**	**OR (95% CI)**
aMMP-8 (μg/L)	**1.002 (1.001–1.003)***	BMI	**0.283 (0.113–0.714)***
		BMI x BMI	**1.030 (1.009–1.052)***
BOP (%)	**1.024 (1.0003–1.049)***	Education level {1, 2}, reference = 2	1.407 (0.592–3.345)
Gender (boy)	**5.047 (2.168–11.750)****	IgM (μg/L)	0.949 (0.837–1.076)
IgG (μg/L)	**0.980 (0.963–0.997)***	Toothbrushing (times per week)	0.929 (0.828–1.043)
Pack-years of smoking	**1.090 (1.008–1.179)***	*T.d.*	1.268 (0.504–3.187)
RC (%)	0.963 (0.886–1.045)	*T.f.*	1.875 (0.762–4.616)
Interaction effects	**OR (95% CI)**		
BOP% × RC%	**1.002 (1.0001–1.003)***		
Education level {1, 2} × gender {1, 2} × toothbrushing (times per week)	**1.064 (1.015–1.115)***		

OR, odds ratio; CI, confidence interval.

Codings: Dependent variable in the model was based on four groups of the number of ≥ 4mm periodontal pockets: zero pockets = 0, 1–3 pockets = 1, 4–7 pockets = 2 and more than 7 pockets 3. Risk factors in the model were BOP, bleeding on probing; RC, root calculus; VPI, visual plaque index. BMI = body mass index; Education level = {1, basic level; 2, upper secondary level}; gender = {1, boy; 2, girl}. T.d. = Treponema denticola; T.f. = Tannerella forsythia. Bold indicates significance at P < 0.05: * P value < 0.05; ** P value < 0.001.

Table 2. *Diagnostic accuracy of active MMP-8 (aMMP-8) point-of-care (PoC) and bleeding on probing (BOP 20%) (cutoff 20% for positives) tests among Finnish adolescents from the city of Kotka (N = 47) by the four initial periodontitis or subclinical periodontitis cases (PD1–PD4). The table is reproduced and modified from Räisänen et al (2019, Diagnostics) under the terms and conditions of the Creative Commons Attribution (CC BY) license http://creativecommons.org/licenses/by/4.0/.*

	Periodontal Status		Measures of the Effectiveness of a Diagnostic Test							
Test	**PD1**	**Healthy**	**OR**	**Se**	**Sp**	**Acc**	**FN**	**FP**	**AUC**	**MCC**
aMMP-8 PoC+	14	0	34.6	48.30 %	100.00 %	68.10 %	45.50 %	0.00 %	0.741	0.51
aMMP-8 PoC−	15	18								
BOP 20%+	7	0	12.3	24.10 %	100.00 %	53.20 %	55.00 %	0.00 %	0.621	0.33
BOP 20%−	22	18								
Test	**PD2**	**Healthy**	**OR**	**Se**	**Sp**	**Acc**	**FN**	**FP**	**AUC**	**MCC**
aMMP-8 PoC+	14	0	63.1	63.60 %	100.00 %	80.00 %	30.80 %	0.00 %	0.818	0.66
aMMP-8 PoC−	8	18								
BOP 20%+	7	0	17.9	31.80 %	100.00 %	62.50 %	45.50 %	0.00 %	0.659	0.42
BOP 20%−	15	18								
Test	**PD3**	**Healthy**	**OR**	**Se**	**Sp**	**Acc**	**FN**	**FP**	**AUC**	**MCC**
aMMP-8 PoC+	13	0	111	76.50 %	100.00 %	88.60 %	18.20 %	0.00 %	0.882	0.79
aMMP-8 PoC−	4	18								
BOP 20%+	6	0	20.9	35.30 %	100.00 %	68.60 %	37.90 %	0.00 %	0.676	0.47
BOP 20%−	11	18								
Test	**PD4**	**Healthy**	**OR**	**Se**	**Sp**	**Acc**	**FN**	**FP**	**AUC**	**MCC**
aMMP-8 PoC+	12	0	102.8	75.00 %	100.00 %	88.20 %	18.20 %	0.00 %	0.875	0.78
aMMP-8 PoC−	4	18								
BOP 20%+	6	0	22.9	37.50 %	100.00 %	70.60 %	35.70 %	0.00 %	0.688	0.49
BOP 20%−	10	18								

aMMP-8 PoC+ = PerioSafe® test positive; aMMP-8 PoC− = PerioSafe® test negative; BOP 20%+ = bleeding on probing over 20%; BOP 20%− = bleeding on probing less than or equal 20%. PD1 = the number of adolescents that have at least one ≥4 mm periodontal pocket; PD2 = the number of adolescents that have at least two ≥4 mm periodontal pockets; PD3 = the number of adolescents that have at least three ≥4 mm periodontal pockets; PD4 = the number of adolescents that have at least four ≥4 mm periodontal pockets; Healthy = the number of adolescents that do not have any ≥4 mm periodontal pockets. OR = diagnostic odds ratio (Haldane–Anscombe correction). Se = sensitivity; Sp = specificity; Acc = accuracy; FN = the percentage of false negatives; FP = the percentage of false positives; AUC = the area under the receiver operating characteristic curve; MCC = Matthews correlation coefficient.

4.3 BACTERIAL CHALLENGE CONFOUNDS THE ASSOCIATION BETWEEN BLEEDING ON PROBING AND INITIAL PERIODONTITIS (STUDY III)

The relative impact of local and modifying factors on BOP propensity was studied among 544 adolescents aged 15-17 (see Table 3). Dental plaque had clearly the strongest impact on BOP propensity, much stronger compared to the extent of periodontal pocketing, root calculus or salivary aMMP-8 levels when adjusted for these factors and smoking and gender (Table 3). In that regard, there were several adolescents with many ≥ 4mm periodontal pockets and relatively low BOP levels, but also several adolescents with no periodontal pocketing and high BOP levels.

Table 3. *The relative impact (adjusted for other variables) of local and modifying factors on bleeding on probing propensity assessed by standardized beta coefficients and t-values. The larger the absolute value of t-value or standardized beta coefficient is, the larger the impact is.*

Local or modifying factor	Standardized beta coefficient	*t-value*
Visual plaque index (dental plaque)	.369	*8.74*
Toothbrushing	−.158	*−4.001*
The number of periodontal pockets (≥ 4mm)	.153	*3.91*
Root calculus	.124	*2.833*
Salivary aMMP-8 (IFMA)	.072	*2.014*
Gender (girl)	*.050*	*0.191*

4.4 MOUTHRINSE IN AMMP-8 TESTING IMPROVES THE DIAGNOSTIC ACCURACY IN COMPARISON TO SALIVA (STUDY IV)

In this study, the salivary and mouthrinse concentrations of aMMP-8 were measured by IFMA and the aMMP-8 PoC mouthrinse test, respectively, utilizing the same antibody. They were compared against the ≥ 4mm periodontal pocket status among 47 Finnish adolescents from the city of Kotka (see Figure 4). The aMMP-8 PoC mouthrinse test gave no false positives, while there were a few unexpectedly high salivary aMMP-8 levels that were aMMP-8 PoC mouthrinse test negatives (< 20 ng/ml). Although both, the salivary and mouthrinse aMMP-8 levels, are associated with initial periodontitis, the strength of association seems stronger when using mouthrinse.

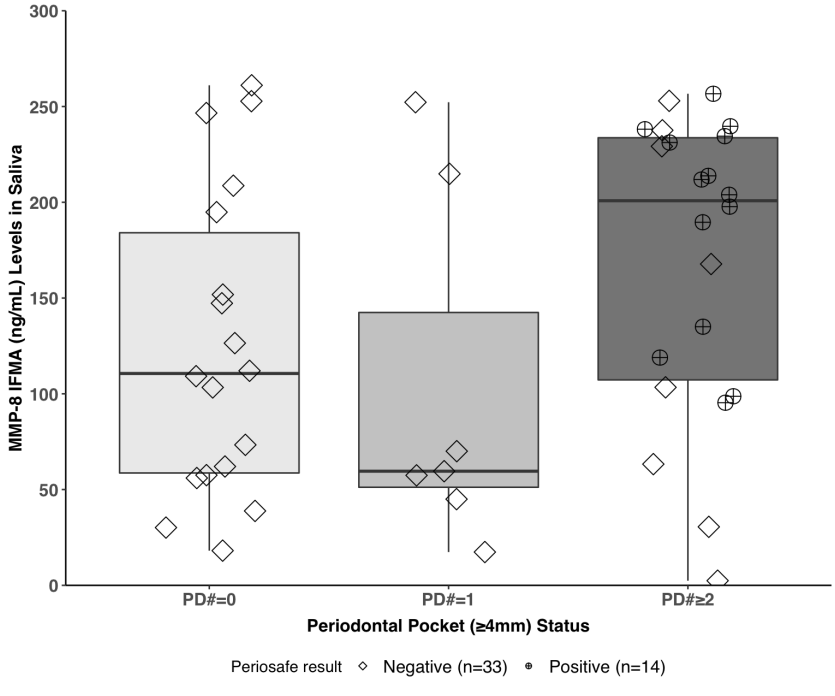

Figure 4 Salivary aMMP-8 IFMA (ng/mL) levels against periodontal pocket (≥4 mm) status (zero [PD#=0], one [PD#=1], and at least two [PD# ≥ 2] periodontal pockets) where IFMA levels are categorized by the corresponding aMMP-8 PoC test result measured from oral mouthrinse.

5 DISCUSSION

5.1 AMMP-8 AND OTHER RISK FACTORS FOR INITIAL PERIODONTITIS

This thesis study is in agreement with the findings of previous studies on the role of bacterial biofilm and its dysbiosis as an etiological risk factor for the initiation and progression of (initial) periodontitis (Colombo & Tanner, 2019; Hajishengallis et al., 2020; Van Dyke et al., 2020). The bacterial biofilm can first trigger gingivitis and then further lead to gingival pocket formation if left untreated. Bacterial biofilm alone seems not sufficient for the development of periodontitis. To convert gingivitis to periodontitis there is also required an ineffective, dysregulated, and destructive host response that results from the interaction between the bacterial biofilm and the host (Hajishengallis et al., 2020).

Conversion from gingivitis to periodontitis is not a visible process to patients. It is usually detected only in dental examination when visible signs of periodontitis emerge, i.e., clinical attachment loss (CAL), deepened gingival pockets, and radiological alveolar bone loss. In that regards, oral fluid biomarkers have been extensively researched to identify potential biomarkers for the onset of periodontitis (Kinane et al., 2017; Ghallab et al., 2018; Arias-Bujanda et al., 2019; Arias-Bujanda et al., 2020; Kc et al., 2020; Gellibolian et al., 2022). Previous studies have found that an increase in the oral fluid aMMP-8 levels to pathological levels is associated with periodontal degradation and progression of periodontitis (Lee et al., 1995; Mancini et al., 1999; Romanelli et al., 1999; Kiili et al., 2002; Mäntylä et al., 2003; Mäntylä et al., 2006; Sorsa et al., 2010; Leppilahti et al., 2011; Leppilahti et al., 2014a; Leppilahti et al., 2014b; Izadi Borujeni et al., 2015; Leppilahti et al., 2015; Johnson et al., 2016; Sorsa et al., 2016; Lorenz et al., 2017; Alassiri et al., 2018; Deumer et al., 2019; Grigoriadis et al., 2019; Schmalz et al., 2019; Taylor et al., 2019; Sorsa et al., 2020; Keles Yucel et al., 2020; Lähteenmäki et al., 2020; Hernández et al., 2021; Öztürk et al., 2021; Deng et al., 2022; Umeizudike et al., 2022). Similarly and in agreement with these studies, in this thesis, elevated aMMP-8 concentrations in both saliva and mouthrinse were associated with and were reflected as periodontal pocketing and risk for initial periodontitis.

Although previous studies have reported an association between periodontitis and an increase in the levels of PMN elastase (that also activates MMP-8) (Ingman et al., 1994; Nizam et al. 2014; Arias-Bujanda et al., 2019), this thesis found no significant association between PMN elastase and initial periodontitis in adolescents. This result is also in agreement with the recent findings of the study by Raivisto et al (2020). This suggests that PMN elastase has lesser role in initial periodontitis compared with aMMP-8. Interestingly,

this thesis found that elevated total salivary IgG levels were associated with lower risk of initial periodontitis in adolescents. However, previous studies have found contradicting results in this regard, which may be due to differences in saliva collection methods (Aufricht et al., 1992; Kaufman et al., 2000; Naiff et al., 2014).

A traditional clinical periodontal parameter BOP was also associated with periodontal pocketing and initial periodontitis but not as strongly as aMMP-8. The aMMP-8 levels measured by the aMMP-8 PoC mouthrinse test was diagnostically more accurate (measured by accuracy, AUC and MCC) than BOP to detect signs of initial periodontitis in adolescents (Räisänen et al., 2019a). The sensitivity of aMMP-8 PoC test for initial periodontitis was at least two times greater compared with BOP (Räisänen et al., 2019a). In addition, all the adolescents that had 6–32 periodontal pockets of at least 4mm were detected correctly by aMMP-8 PoC test, while BOP was less accurate here (Räisänen et al., 2019a). Thus, the results support the utility of aMMP-8 and aMMP-8 PoC testing to identify potential adolescents at risk of initial periodontitis.

This thesis showed that gingival bacterial biofilm may confound and weaken the association between BOP and initial periodontitis in adolescents (Räisänen et al., 2021a). The level of oral hygiene (dental plaque and root calculus) had the largest relative impact on the propensity of BOP compared with the extent of periodontal pocketing, salivary aMMP-8 levels (i.e., risk of active collagenolysis), smoking, toothbrushing or gender. As Trombelli et al (2018) state, BOP levels are an important signal of the extent of gingival inflammation rather than the severity of periodontal inflammation. In that regard, it was noted that although the likelihood of gingival bleeding increased with increasing number of periodontal pockets, the impact of the oral hygiene level was so strong that BOP levels could be low, even on a healthy level, even if an adolescent had clear signs of initial periodontitis (several ≥ 4 mm deep periodontal pockets) (Räisänen et al., 2021a). Likewise, a poor oral hygiene (high levels of bacterial biofilm) would appear to increase the amount of gingival bleeding regardless of the number of periodontal pockets (Räisänen et al., 2021a). Therefore, the level of oral hygiene and the amount of bacterial biofilm would appear to be eventually the main determinant of the degree of gingival bleeding instead of active collagenolysis (aMMP-8 levels) (Räisänen et al., 2021a). These findings may also explain at some degree the results from previous BOP studies, such as the poor sensitivity of BOP to the progression of periodontitis (Lang et al., 1990; Joss et al., 1994).

Although BOP levels may potentially predispose to erroneous conclusions about the periodontal condition and its severity in adolescents, BOP levels should not be disregarded in periodontal diagnostics. They are an important indicator of the extent of bacterial challenge and the current need for reducing and controlling this challenge to prevent its adverse effects on the gingival inflammation and potential progression of bacterial dysbiosis (Trombelli et al., 2018). This thesis study found that partly calcified bacterial biofilm may be

particularly harmful for periodontal health in presence of elevated BOP levels (Heikkinen et al., 2019). Formation of calculus takes time and during this process the bacterial biofilm has had more time to irritate the gingival tissues and cause gingival inflammation. The longer the situation persists, the more likely it is to reduce the recovery capacity of tissues which increases the risk of initiation of tissue collagen breakdown leading to gingival pocket formation and initial periodontitis (Löe et al., 1965; Theilade et al., 1966; Manji et al., 2018).

This thesis found that smoking has a harmful, dose-dependent (i.e., pack-years of smoking) effect in oral cavity among adolescents. This result is in agreement with and further extends previous studies (Heikkinen et al., 2008; Rosa et al., 2008; Heikkinen et al., 2010; Brook, 2011; Kumar et al., 2011; Heikkinen et al., 2012; Wu et al., 2016; Karasneh et al., 2017; Leite et al., 2018; Freitag-Wolf et al., 2019; Šutej et al., 2021). Smoking seems to play a significant role here with its many toxic compounds that may disturb the microcirculatory, inflammatory and immune systems and host's response to bacterial challenge (Lee et al., 2012; Nociti et al., 2015). That may impact not only on the recovery capacity of tissues but also potentially modifies bacterial biofilm into more pathogenic and more dysbiotic state both in adults and adolescents that could initiate periodontitis (Heikkinen et al., 2008; Heikkinen et al., 2010; Brook, 2011; Kumar et al., 2011; Heikkinen et al., 2012; Wu et al., 2016; Karasneh et al., 2017). Moreover, two bacteria of the red complex, *T.d.* and *T.f.*, were found to be risk factors in the univariable analysis of this thesis study; however, in the multivariable analysis when adjusted for other variables the two bacteria were identified as confounders interacting with both the dependent and independent variables in the model. For example, chymotrypsin-like-proteinase of *T.d.* is known to be able to activate latent proMMP-8 to aMMP-8 linking *T.d.* and aMMP-8 (Gürsoy et al., 2018; Petain et al., 2021). Previous studies have found several different putative periodontal bacteria associated initial periodontitis, including *T.d.* and *T.f.*, but in different putative bacteria subsets (Albandar et al., 1997; Suda et al., 2004; Tanner et al., 2006). Thus, it seems that the dysbiosis plays more important role than a single bacteria species in the initiation of periodontitis.

At the individual level, male gender was a risk factor for initial periodontitis. This may be related to differences in the health behaviors of girls and boys, such as smoking and toothbrushing, but also to gender differences in the host response to the bacterial challenge. Previous studies support that there are sexual dimorphisms in innate and adaptive immune systems due to, for example, genetic and gene regulation differences between genders (Shiau & Reynolds, 2010; Valerio et al., 2017). They may lead to stronger local inflammatory response to bacterial infection that plays a role in the differences in the susceptibility to periodontitis between females and males (Shiau & Reynolds, 2010; Valerio et al., 2017). In that regard, for example, Heikkinen et al (2016b) have found a link between initial periodontitis and genetic polymorphisms of pro-inflammatory mediators MMP3, CD28, and VDR. In

this thesis study, toothbrushing by itself did not appear to be associated with the formation of gingival pockets in the logistic regression analysis model, perhaps because toothbrushing is linked to the level of bacterial biofilm that was already utilized in the model. However, a significant interaction effect of toothbrushing, gender and education level was found. This suggests differences in toothbrushing habits (e.g., length, precision of brushing) between boys and girls at the different education levels. According to the Finnish school health surveys in 2019 and 2021, boys in the upper comprehensive school, high school and vocational school brush their teeth less often than girls (National Institute for Health and Welfare of Finland). The same observation was made in this study. But toothbrushing was clearly the lowest among boys studying in the vocational school, which is also in agreement with the recent Finnish school health surveys (National Institute for Health and Welfare of Finland). Thus, there seems to be individual differences in oral home care habits and skills, which differ slightly at different school levels and put the young people with a lower level of oral hygiene at greater risk for both initial periodontitis and cariological problems.

Previous studies have shown an association between periodontitis and obesity in adults, adolescents and children (Khan et al., 2018; Jepsen et al., 2020; Tegelberg et al., 2021). In agreement with these findings and extending them, this thesis study found not only that overweight but also underweight are associated with periodontal pocketing and initial periodontitis. Both overweight and underweight may indicate potential risk for health, nutritional, or metabolic problems having association with several non-communable disorders (Golubnitschaja et al., 2021). Furthermore, dietary differences especially in the use of sugar can contribute not only to body weight but also to the amount of subgingival and supragingival bacterial biofilm predisposing to periodontal and cariological diseases. Thus, the best ways to prevent these oral health problems is dental home care and dental care by a dentist / oral hygienist.

5.2 AMMP-8 POC TESTING AND PERIODONTAL DISEASE DIAGNOSTICS

Oral fluid biomarkers have been a target of extensive research in periodontology during the last 30 years (Ghallab, 2018; Arias-Bujanda et al., 2019; Arias-Bujanda et al., 2020; Gul et al., 2020; Kc et al., 2020). One of the most promising and successful oral fluid biomarkers for periodontitis is neutrophil collagenase i.e., MMP-8. A prospective study by Lee et al (1995) was one of the first to demonstrate that elevated neutrophil collagenase activity (aMMP-8) is strongly associated with the progressing periodontal connective tissue breakdown and periodontitis. Since then, several studies have shown similar results that the elevated active MMP-8 levels in saliva, GCF and mouthrinse associate with periodontal clinical parameters, CPITN

index, and disease progression of periodontitis (Mancini et al., 1999; Romanelli et al., 1999; Kiili et al., 2002; Mäntylä et al., 2003; Mäntylä et al., 2006; Sorsa et al., 2010; Leppilahti et al., 2011; Leppilahti et al., 2014a; Leppilahti et al., 2014b; Nwhator et al., 2014; Izadi Borujeni et al., 2015; Leppilahti et al., 2015; Heikkinen et al., 2016a; Heikkinen et al., 2016b; Johnson et al., 2016; Heikkinen et al., 2017a; Heikkinen et al., 2017b; Lorenz et al., 2017; Alassiri et al., 2018; Deumer et al., 2019; Grigoriadis et al., 2019; Schmalz et al., 2019; Taylor et al., 2019; Sorsa et al., 2020; Keles Yucel et al., 2020; Lähteenmäki et al., 2020; Raivisto et al., 2020a; Raivisto et al., 2020b; Raivisto. 2020c; Deng et al., 2021a; Hernández et al., 2021; Öztürk et al., 2021; Deng et al., 2022; Heikkinen et al., 2022; Umeizudike et al., 2022). Noteworthily, those studies have utilized different aMMP-8 detection methods, such as aMMP-8 POCT, IFMA, DentoELISA, and SAW biosensor, that have been shown to correlate well with each other, particularly when used with the same aMMP-8 specific monoclonal antibodies (Sorsa et al., 2010; Leppilahti et al., 2014b; Nieminen et al., 2015; Lorenz et al., 2017; Alassiri et al., 2018).

In agreement with the previous aMMP-8 studies of periodontitis, this thesis study found a significant association between aMMP-8 and initial periodontitis. The strength of association between aMMP-8 and initial periodontitis was several times stronger than the association between BOP 20% (cut-off 20% for positives) and initial periodontitis. Furthermore, the diagnostic accuracy for the aMMP-8 PoC test was much better than BOP 20% suggesting that utilizing aMMP-8 instead of BOP could increase the targeting of the patient treatment. This may be explained by the strong association between the propensity of BOP and adolescent's oral hygiene situation (the levels of dental plaque and calculus). Dental plaque and calculus were the main contributors to the BOP levels, while the extent of periodontal pocketing, aMMP-8 levels, smoking, toothbrushing, or gender had much smaller impact as has already been discussed in section 5.1.

Periodontal disease diagnostics has traditionally relied on BOP in the assessment of gingival inflammation, disease activity of periodontitis and its risk of progression (Lang et al, 2015). However, BOP with its limitations is no longer used in the diagnosis of periodontitis in the 2017 classification system of periodontal diseases but is the main determinant of diagnosing gingivitis (Tonetti et al., 2018; Chapple et al., 2018; Trombelli et al., 2018). Previous studies have shown that BOP is one of the earliest signs of gingivitis preceding redness and swelling that are the visual signs of gingival inflammation (Lang & Bartold, 2020). But although continuous absence of BOP has predicted well periodontal health, the presence of BOP has a low sensitivity for disease progression (Lang et al., 1990; Joss et al., 1994). The results in this thesis study are in agreement with these previous findings. BOP levels reflect mainly the extent of bacterial challenge and gingival inflammation and much less the severity of the periodontal inflammatory condition. Thus, BOP levels may not always be reliable measure for the need of periodontal treatment in

adolescents and factors affecting bleeding on probing propensity should be carefully considered. It should be noted that BOP is associated with the increased capillary fragility due to gingival inflammation (Lang & Bartold, 2020), while aMMP-8 is a neutrophil derived activated enzyme that plays a key role in the periodontal tissue destruction cascades (Cui et al., 2017, Sorsa et al., 2006; Sorsa et al., 2016). Neutrophils maintain periodontal health and homeostasis but also play an important role in periodontal immunity as the first line of defense against microbial challenge (Scott & Krauss, 2012; Moutsopoulos & Konkel, 2018; Hajishengallis et al., 2020; Uriarte & Hajishengallis, 2022). Thus, aMMP-8 levels seem to associate with the neutrophil activity and better reflect the pathological periodontal inflammation than BOP.

The latest classification system of periodontitis described in Tonetti et al (2018) defines the diagnosis of periodontitis as based on clinical measurements of periodontal diagnostics using periodontal probe including CAL, probing depth and furcation involvement, as well as radiographic measurements of bone loss. They mainly provide retrograde information about the severity and extent of periodontitis, which underscores the importance of developing new methods and measures to increase the accuracy of assessing and monitoring of the disease activity of periodontitis and its risk of progression (Kinane et al., 2017; Tonetti et al, 2018; Gellibolian et al., 2022). With the currently utilized traditional periodontal diagnostics the identification of initial periodontitis is eventually affected by the experience and skills of the operator with periodontal probing potentially causing misclassifications of the disease (Tonetti et al., 2018). In that regard, the 2017 classification system of periodontitis was built to allow future updates and implementation of oral fluid biomarkers. Sorsa et al (2020) was the first to show a significant association between aMMP-8 POCT (mouthrinse) and staging and grading of periodontitis (Tonetti et al., 2018). Their result was further confirmed with aMMP-8 IFMA (GCF) and aMMP-8 POCT (mouthrinse) analysis by Özturk et al (2021). However, these two studies were only partly confirmed by a recent study by Deng et al (2021a). They found that aMMP-8 POCT was associated with periodontitis and periodontal clinical parameters but not stage and grade of periodontitis. A comparison between the study populations in Deng et al (2021a) and Sorsa et al (2020) (Hong Kong and Greece, respectively) showed that the Hong Kong population seemed to have less gingival inflammation (BOP) and lower mean periodontal probing depth than the Greek population (Sorsa et al., 2021). As the aMMP-8 POCT is measuring collagenase activity and disease activity, this is a plausible explanation for the difference between the results in the Hong Kong and Greece populations (Sorsa et al., 2021; Deng et al., 2021b). In that regard, Deng et al (2021a) were able to markedly improve the aMMP-8 POCT diagnostic performance by combining it with periodontitis risk factors (age and smoking). This suggests that combining patient characteristics and aMMP-8 POCT can be beneficial and increase the accuracy of at-risk patient

identification – particularly in patients with periodontitis that is not in active phase. Furthermore, recent studies have shown that oral fluid biomarkers in adjunct with an interview/questionnaire can increase the precision of identification of asymptomatic and undiagnosed periodontitis patients (Verhulst et al., 2019; Räisänen et al., 2021b). Similarly, combining more than one oral fluid biomarkers, such as IL-6 and MMP-8, or aMMP-8 and TREM-1, may be beneficial and result in better diagnostic accuracy (Kc et al., 2020; Räisänen et al., 2020).

5.3 POTENTIAL PITFALLS IN THE INTERPRETATION OF AMMP-8 STUDIES

Lee et al (1995) were one of the first to show that the elevation of active neutrophil collagenase (aMMP-8) is associated with progressive bone loss, but they also found that the elevation of latent neutrophil collagenase is not. That supports the importance of utilizing such antibodies that are sensitive and specific to detect neutrophil collagenase activity (aMMP-8) in periodontitis diagnostics instead of latent and/or total MMP-8 species. This seems a plausible explanation why some MMP-8 studies have found inconsistent results in the periodontitis diagnostics: they have used MMP-8 technologies utilizing antibodies that detect total or mainly latent species of MMP-8 instead of aMMP-8 (Verhulst et al., 2019; Karatas et al., 2020; Romero-Castro et al., 2020; Petain et al., 2021). The different technologies used in the studies may also have an impact on the results, but not as large as the impact of inefficient antibodies. Another plausible explanation may be the type of oral fluid used in the MMP-8 studies. Although elevated aMMP-8 levels in saliva and mouthrinse are associated with periodontitis, in this thesis, it was found that mouthrinse seems to provide a better accuracy for detecting neutrophil collagenase activity (aMMP-8) compared with saliva among adolescents. The result is in agreement with other periodontitis diagnostics studies in adult populations (Sorsa et al., 2020; Katsiki et al., 2021; Deng et al., 2022).

Oral fluids (saliva, GCF and mouthrinse) are readily available to be collected non-invasively without causing bacteremia to the patients unlike when measuring BOP (Olsen, 2008; Gul et al., 2020). The difference between saliva and mouthrinse is in their contents. The mouthrinse collection technique is basically collection of GCF from the whole mouth, while saliva contains not only GCF but also many other products from the salivary glands, epithelial cells and other sources (Drouin et al., 1988; Gangbar et al., 1990; Proctor, 2016; Ghallab, 2018). It has been shown that MMP-8 content in mouthrinse is derived from neutrophils (Gangbar et al., 1990; Sorsa et al., 1992), which makes mouthrinse the representative sampling technique to measure the whole mouth neutrophil collagenase activity (i.e., aMMP-8 levels) from the sites of periodontal inflammation. Saliva on the other hand has a great variety of different components, such as TIMPs, that may suppress the

collagenase activity and decrease salivary aMMP-8 levels (Drouin et al., 1988; Proctor, 2016; Ghallab, 2018). Measurements of salivary biomarker concentration may also be influenced by several patient related factors, such as xerostomia/hyposalivation and viscoelastic properties (Deng et al., 2022). Mouthrinse collection is not affected by the variability in salivary flow rate and volume (Deng et al., 2022), which provides much needed calibration and standardization at the patient level for the whole mouth aMMP-8 testing. Therefore, the impact of the selection of correct antibodies and oral fluid type is potentially large on the diagnostic accuracy of aMMP-8 testing and should be carefully considered when assessing the different MMP-8 studies and the diagnostic utility of aMMP-8. Combining all MMP-8 studies into the same category is likely to cause inaccurate conclusions. This is likely to apply to other oral fluid biomarkers, as well. More research is still required in that area to extend our knowledge. It is worth noting that the aMMP-8 PoC test used in this thesis is a mouthrinse test and not a salivary test.

5.4 AMMP-8 POC TESTING IN THE DIAGNOSIS AND PREVENTION OF INITIAL PERIODONTITIS

It is essential to identify patients at risk of initial periodontitis as early as possible to efficiently target the individual disease prevention and early care. The process should be based on clinical examination and assessment of the patient's individual and environmental risk factors for periodontitis as well as patient's disease activity. Traditional periodontal clinical measurements include periodontal probing depth, CAL, and X-rays to identify signs of history of the disease, its severity and extent as well as complexity of managing patient's disease (Tonetti et al., 2018). In the initial phase of the disease, the severity of periodontitis related tissue destruction is still low or non-existent, but local complexity factors (mainly probing depths ≥ 4mm) and their extent can influence on the treatment choices and experience needed (periodontist, dentist or dental hygienist) to treat the patient (Tonetti et al., 2018; Sanz et al., 2020). Patient's current disease activity of periodontitis alerts from the increased risk of ongoing destruction of the connective tissue and disease progression, which plays a key role in identifying and monitoring the initiation and progression of periodontitis (Gellibolian et al., 2022). Thus, it should be combined with other measures of risk for future progression, namely evidence of past disease progression and grade modifiers (smoking and diabetes), which could lead to more accurate assessment of total risk of disease progression (Tonetti et al., 2018; Gellibolian et al., 2022).

Together with risk factors for periodontitis, most importantly smoking and diabetes, periodontal clinical measurements provide some indirect information to a clinician about patient's risk of the initiation of periodontitis and its progression as well as patient's expected response to periodontal treatment (Tonetti et al., 2018; Gellibolian et al., 2022). For example, smokers

may respond less optimally to treatment especially the patients with complexity factors, and thus they require more frequent maintenance treatment and prevention (Ah et al., 1994; Heasman et al., 2006). Disease activity measured by αMMP-8 PoC mouthrinse test can be regarded as a direct measure of active collagenolysis and potential risk of ongoing periodontal breakdown. A positive αMMP-8 PoC mouthrinse test result, particularly if it is repeatedly positive, indicates an increased risk of active collagen degradation in the periodontium (Lee et al., 1995; Mancini et al., 1999; Romanelli et al., 1999; Kiili et al., 2002; Mäntylä et al., 2003; Mäntylä et al., 2006; Sorsa et al., 2010; Leppilahti et al., 2011; Leppilahti et al., 2014a; Leppilahti et al., 2014b; Nwhator et al., 2014; Izadi Borujeni et al., 2015; Leppilahti et al., 2015; Heikkinen et al., 2016a; Heikkinen et al., 2016b; Johnson et al., 2016; Heikkinen et al., 2017a; Heikkinen et al., 2017b; Lorenz et al., 2017; Alassiri et al., 2018; Deumer et al., 2019; Grigoriadis et al., 2019; Schmalz et al., 2019; Taylor et al., 2019; Sorsa et al., 2020; Keles Yucel et al., 2020; Keskin et al., 2020; Lähteenmäki et al., 2020; Raivisto et al., 2020a; Raivisto et al., 2020b; Raivisto. 2020c; Deng et al., 2021a; Hernández et al., 2021; Öztürk et al., 2021; Deng et al., 2022; Heikkinen et al., 2022; Umeizudike et al., 2022). A negative aMMP-8 PoC test can be interpreted that there is no evidence of active collagen degradation and risk for progression of connective tissue breakdown is on a safe level. Furthermore, comparable results to periodontitis studies have been published regarding peri-implantitis, where elevated aMMP-8 levels in PISF have been reported among peri-implantitis patients (Alassiri et al., 2018; Golub et al., 2020; Lähteenmäki et al., 2022b; Xanthopoulou et al., 2022). Another recent study found in a longitudinal data that aMMP-8 levels in PISF has good reliability to predict future peri-implantitis in dental implants (Guarnieri et al., 2022).

Table 4 provides an example of how oral health care professionals could assess their patients' personalized need for prevention and intervention (health counseling, dental home care instructions etc.) as well as their need for anti-infective treatment and shorter recall intervals for maintenance therapy. In that regard, multidimensional periodontitis risk assessment tools have been previously studied and reported (Lang et al., 2015; Ferraiolo, 2016). In a similar fashion, the different pieces of information available for the clinician can be categorized into no or low risk, moderate risk, and high risk groups as in Table 4. The points are added together to calculate the total risk to categorize, compare and prioritize patients according to their need of prevention and treatment of periodontitis. Future studies in that regard are still required to determine the best decision variables and their optimal weights of the decision variables.

Table 4. *A multidimensional model to assess risk for initial periodontitis. Total risk is calculated by adding all the risk points together.*

Variables	No or low risk (0 points)	Moderate risk (1 points)	High risk (2 points)
Clinical attachment loss /radiographic bone loss	No	Low	Moderate/high
Periodontal pocket depth (PPD)	No	PPD = 4-5mm	PPD ≥ 6mm
Extent of periodontal pockets	No	Localized or molar-incisor pattern	Generalized
Evidence of disease progression	No	Yes, only localized progression	Yes, progression from localized to generalized
Current bacterial challenge and oral hygiene situation	Low BOP % and little or no plaque/calculus	Elevated BOP % and little or no plaque/calculus	High BOP % and plaque/calculus levels
Smoking	No	< 10 cigarettes/day	≥ 10 cigarettes/day
Diabetes	No	Diabetes with HbA1c < 7.0 %	Diabetes with HbA1c ≥ 7.0 %
Disease activity	aMMP-8 PoC testnegative	aMMP-8 PoC test positive	Repeatedly aMMP-8 PoC test positive

As the results in this thesis suggest, elevated aMMP-8 levels measured by the aMMP-8 PoC test seem more accurate/reliable biomarker for initial periodontitis compared to BOP levels. As such, a positive aMMP-8 PoC test may be a concerning sign for not only for patients with periodontitis but also adolescents with healthy periodontium or gingivitis. As Heikkinen et al (2022) recently pointed out there may be one or more precedent stages before stage I of periodontitis (initial periodontitis), such as subclinical stage (Raivisto et al., 2020c). Here, a positive aMMP-8 PoC test result may be regarded as an initial alarming factor in those early precedent stages that could be used for assessing the potential risk of initiation and progression of periodontal destruction (collagenolysis) in adolescents. In that regard, two recent studies using Western blot analysis showed that active forms of MMP-8 are not found in the saliva of gingivitis patients during the gingival inflammation and its resolution, but they exist in the saliva of periodontitis patients (Gürsoy et al., 2018; Silbereisen et al., 2020). This suggests that aMMP-8 PoC test may be useful in detecting the transformation of gingivitis to periodontitis. Another

recent study showed that aMMP-8 PoC test positive adolescents with a healthy periodontium or gingivitis can be treated to test negatives with intensified oral hygiene instructions and anti-infective periodontal treatment (Raivisto et al., 2020b). That suggest that the disease may still be reversible in the initial stages of periodontitis. Similar measures should be taken in adolescents with stage I periodontitis (initial periodontitis) while monitoring their response to the treatment (Sanz et al., 2020).

5.5 STRENGTHS AND LIMITATIONS

Like every study, this thesis study has its strengths and limitations. The strength of this study was the two birth cohorts of systemically healthy, untreated 15-17-year-old adolescents that were not using medications and were without a long treatment history. Furthermore, the same periodontist performed their clinical oral health examination for all these adolescents, and a wide range of data was collected and analyzed. Recruiting adolescents (or children) to any study is always challenging and time-consuming, and the same usually applies to obtaining research permits. That may explain why the majority of the studies of periodontitis and its risk factors are in adult populations and the studies in adolescent (or children) populations are in the minority. The sample size in this thesis study was at least sufficient in the Studies I and III but relatively small in the Studies II and IV. However, it should be noted that statistical significance was found even in the study II suggesting that the power in the studies was large enough.

A potential limitation is that this thesis study used PPD instead of CAL. However, the participants were adolescents that were diagnosed between periodontally healthy and having initial periodontitis meaning that they had only a short periodontal history if any. It can be argued if the difference between PPD and CAL had any meaningful effect to the results in this study. For example in the Study I, adolescents were grouped according to the number of sites with PPD ≥ 4mm creating an ordinal variable representing the deterioration of periodontal health towards initial periodontitis (as discussed in section 3.6). Interestingly, probing depth was positively associated with this ordinal variable among the adolescents but radiographic attachment loss was not. The amount of the radiographic attachment loss is usually still low or non-existent at their age, but some attachment loss was nevertheless detected even among a few adolescents without periodontal pocketing. That suggests that the deterioration of periodontal health may not be dependent on the very early stage of radiographic attachment loss, which makes periodontal pocketing more useful factor to model the first steps to initial periodontitis.

And finally, as this thesis study analyzed cross-sectional data collected at a specific point of time, causality cannot be directly. However, the results even from cross-sectional studies can support previous findings and also help to generate causal hypotheses based on the associations found. The hypotheses

from this thesis should be further studied in longitudinal studies and other studies to determine if causality really exists. It is noteworthy that prospective longitudinal studies are not without limitations either. Mainly because in some cases they can be unethical and thus prohibited. Longitudinal studies usually require also large amounts of time which tends to get expensive.

6 CONCLUSIONS

This thesis study researched risk factors for initial periodontitis and potential benefits of aMMP-8 PoC mouthrinse testing in identifying adolescents at risk of initial periodontitis. The main risk factors for initial periodontitis were elevated aMMP-8 levels, accumulation of dysbiotic biofilm together with elevated levels of bleeding on probing, pack-years of smoking, and male gender. Obesity and underweight were also associated with initial periodontitis and its extent. Furthermore, aMMP-8 PoC mouthrinse test showed stronger association and better accuracy in identifying clinical signs of initial periodontitis than measuring BOP levels. The results suggest that utilizing aMMP-8 test instead of BOP levels could reduce the risk of undertreatment of patients. Bacterial challenge (dental plaque and calculus) has a major impact on BOP levels confounding its association with the clinical signs of initial periodontitis. If the oral hygiene is on a good level, BOP levels can be low even if there are extensive periodontal pocketing. Thus, BOP levels seem to reflect mainly the extent of bacterial challenge and gingival inflammation and much less the severity of the periodontal inflammatory condition. Thus, BOP levels may provide unreliable information about the periodontal treatment need in adolescents, in case the factors affecting bleeding on probing propensity are not carefully considered. Here, aMMP-8 PoC mouthrinse test could be a useful adjunctive tool in periodontal diagnostics to improve the detection of adolescents at risk of initial periodontitis and targeting their disease prevention. On the long term, using aMMP-8 test could potentially help to improve the effectiveness of prevention and reduce the future adverse effects of periodontitis and the need for expensive dental specialist treatment among the at-risk patients. This could reduce the health and economic burdens for these individuals, communities and society caused by periodontitis. Finally, aMMP-8 levels in mouthrinse seems to have stronger association with initial periodontitis than salivary aMMP-8 levels, which supports mouthrinse as the preferred oral fluid for the patient-specific whole-mouth aMMP-8 periodontal disease diagnostics.

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