SERUM AND SALIVA BIOMARKERS IN CARDIOVASCULAR DISEASES AND PERIODONTITIS – SMOKING AS A CONFOUNDING FACTOR

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ACADEMIC DISSERTATION

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ABSTRACT

Atherosclerotic cardiovascular diseases (CVD) and periodontitis—that is, disease in tooth-supporting tissues in the periodontium—are chronic and common diseases worldwide. The rupture of atherosclerotic plaque can lead to acute coronary syndrome (ACS), including unstable angina pectoris (UAP), acute myocardial infarction (AMI), or ischemic stroke. Furthermore, periodontitis is an independent risk factor for CVD. Thus, biomarkers measured from serum and saliva samples may improve the prevention, early diagnosis, and prognosis of these diseases. Using such biomarkers could also improve patient quality of life and reduce healthcare costs, since such biomarkers could be used in the wider population and lead to early diagnosis.

The degradation of the extracellular matrix (ECM) is crucial both in periodontitis and atherosclerotic plaque rupture. Thus, matrix metalloproteinases (MMPs), particularly MMP-8 and MMP-9, as effective enzymes for ECM destruction play important roles in these diseases. For instance, the tissue inhibitor of matrix metalloproteinase (TIMP)-1 is an endogenous inhibitor of MMP-8 and MMP-9, participating in the disease processes of both periodontitis and CVD. By contrast, myeloperoxidase (MPO) is an inflammatory mediator, which can activate MMP-8 and MMP-9 and inactivate TIMP-1. These molecules can be measured from the serum and saliva, and may be used to diagnose both periodontitis and CVD.

While these molecules have long been investigated, all factors affecting their diagnostic and prognostic use remain unexamined. This PhD study aimed to explore MMP-8, MMP-9, TIMP-1, and MPO in the diagnosis of ACS, and to examine serum MMP-9 and TIMP-1 in the prognosis of secondary CVD events. In addition, saliva and serum biomarkers, such as MMP-8, TIMP-1, and MPO, were studied in ischemic stroke patients alongside healthy controls. Moreover, saliva and serum MMP-8, MMP-9, TIMP-1, and MPO were examined in periodontitis and ACS, with the aim of determining if periodontitis affects ACS diagnostics and if cardiac status affects periodontal diagnosis using these biomarkers. Finally, the effect of smoking on serum and saliva biomarkers in ACS and periodontitis was investigated.

This PhD study consisted of three different study populations from which the aforementioned biomarker levels were measured. Firstly, the Triangel data collected at Lund University Central Hospital in Sweden (n = 669) comprised ACS patients and healthy controls. Secondly, the case-control data collected from Ludwigshafen, Germany (n = 198), consisted of ischemic stroke patients...
and their controls whose oral status was estimated through a questionnaire and cumulative risk score (CRS) to assess their risk of having periodontitis determined from saliva molecules. Thirdly, the Parogene cohort (n = 508) consisted of patients with an indication of coronary angiography seen at Helsinki University Central Hospital in Finland. The clinical oral status was examined for patients in this cohort.

The data from this study indicate that saliva biomarkers distinguish periodontitis patients from controls, and cardiac status has a minor effect on the salivary periodontal diagnostics. Serum biomarkers designate the systemic state, and can be utilized in the diagnosis of CVD, including both ACS and ischemic stroke. This observation emerged from the Ludwigshafen study, where elevated serum biomarkers associated with ischemic stroke, while stroke-free healthy controls more frequently exhibited signs of current periodontitis reflected in elevated saliva biomarker levels. However, in the Parogene study periodontitis interfered in the diagnosis of ACS using serum MMP-9, indicating the systemic effect of periodontal disease. Finally, smoking complicates the use of saliva and serum biomarkers, whereby the smoking dimension (i.e., quantity, duration, and time since cessation) influences the biomarkers.

Overall, we found that periodontitis and CVD associate with one another and the degradation of ECM remains crucial in both diseases. Thus, the same biomarkers can be utilized in the diagnosis of both diseases. However, the selection of sample material is crucial. Specifically, saliva is suitable for distinguishing periodontitis patients from controls, whereas serum biomarkers are suitable for CVD diagnostics. Smoking remains a factor that must be taken into account when using both serum and saliva biomarkers, such as MMP-8, MMP-9, TIMP-1, and MPO. To conclude, these biomarkers could be utilized by different healthcare professionals for screening, early diagnosis, and prevention.
ABSTRAKTI


Potentiaalisia parodontiitin ja sydän- ja verisuonisairauksien merkkiaineita ovat matriisin metalloproteinaasit (MMPt), erityisesti MMP-8 ja MMP-9, jotka osallistuvat soluväliaineen hajottamiseen sekä parodontiumissa että aterioskleroottisen plakin repeämisessä. Matriisin metalloproteinaasin kudosestääjä (TIMP)-1 on MMP-8:n ja MMP-9:n sisäsyntyinen (endogeneinen) inhibiittori ja täten TIMP-1 on tärkeä kyseisissä tautitiloissa. Myeloperoksidaasi (MPO) on tulehduksen välittäjä ja voi aktivoida MMP-8:ä ja MMP-9:ä sekä inaktivoida TIMP-1:stä. Näitä molekyylejä voidaan mitata sekä syljestä että seerumista ja hyödyntää sydän- ja verisuonisairauksien ja parodontiitin diagnostiikassa.

Vaikka kyseisiä molekyylejä on tutkittu pitkään, ei kaikkia niiden diagnostiikkaan ja taudin ennusteeeseen (prognostiikkaan) vaikuttavia tekijöitä ole vielä selvitetty. Tämän vääristutkimuksen tarkoituksena oli selvittää MMP-8, MMP-9, TIMP-1 ja MPO käyttökelpoisuutta aterioskleroottisten sydänsairauksien diagnostiikassa ja tarkastella MMP-9 ja TIMP-1 entsyymejä sydänraasauksien sekundaaritapahtumien ennustamisessa. Lisäksi iskeemistä aivoinfarktia sairastavilta potilailta ja heidän verrokkeiltaan tutkittiin syljen ja seerumin biomarkereita ja tarkasteltiin parodontiittia kuvaavien sylkimerkkiaineiden sekä seerumimerkkiaiden ja aivoinfarktia yhteyttä. Väitöskirjan toisessa suuremmassa kokonaisuudessa oli tavoitteena tutkia syljen ja seerumin merkkiaineita (MMP-8, MMP-9, TIMP-1, ja MPO) parodontiittissa ja sydänsairauksissa. Lisäksi tarkoituksena oli selvittää onko parodontiittilla vaikutusta seerumimerkkiaineiden sydänsairausdiagnostiikkaan sekä tutkia vaikuttaako sydänstatus paikallisesti syljen parodontiittia kuvaaviin entsyymeihin. Lopuksi tavoitteena oli tutkia myös tupakoinnin vaikutusta syljen merkkiaineisiin parodontiittissa ja seerumimerkkiaineisiin.
sydänsairauksissa.


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Laura Lahdentausta

Helsinki, November 2018
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


In addition, some unpublished data are presented.

The publications are referred to in the text by their roman numerals. The original publications have been reprinted with the permission of their copyright holders.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABL</td>
<td>Alveolar bone loss</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
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<td>AL</td>
<td>Attachment loss</td>
</tr>
<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<tr>
<td>APMA</td>
<td>4-Aminophenylmercuric acetate</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
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<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CAL</td>
<td>Clinical attachment loss</td>
</tr>
<tr>
<td>CEA</td>
<td>Carotid endarterectomy</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CHF</td>
<td>Chronic heart failure</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Creatine-kinase-MB</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CRS</td>
<td>Cumulative risk score</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability-adjusted life years</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography or electrocardiogram</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FN</td>
<td>False negative</td>
</tr>
<tr>
<td>FP</td>
<td>False positive</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>H-FABP</td>
<td>Heart-type fatty acid binding protein</td>
</tr>
<tr>
<td>HGF</td>
<td>Human gingival fibroblasts</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-methyl-glutaryl-coenzyme A</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>hsCRP</td>
<td>High-sensitivity C-reactive protein</td>
</tr>
<tr>
<td>Hs-cTn</td>
<td>High-sensitivity cardiac troponin</td>
</tr>
<tr>
<td>HT</td>
<td>Hemorrhagic transformation</td>
</tr>
<tr>
<td>IFMA</td>
<td>Time-resolved immunofluorometric assay</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
</tbody>
</table>
IL-1 Interleukin 1
IL-1β Interleukin 1β
LAL Limulus amebocyte lysate assay
LDL Low-density lipoprotein
Lp-PLA2 Lipoprotein-associated phospholipase A2
LPS Lipopolysaccharide
LuSST Ludwigshafen Stroke Study
LV Left ventricular
MACE Major adverse cardiovascular event
MI Myocardial infarction
MMP Matrix metalloproteinase
MPO Myeloperoxidase
NADPH Nicotinamide adenine dinucleotide phosphate
NGAL Neutrophil gelatinase-associated lipocalin
NIHSS National Institutes of Health Stroke Scale
NK Natural killer
NO Nitric oxide
NS Not significant
OR Odds ratio
PAD Peripheral artery disease
PBMC Peripheral blood mononuclear cell
PCR Polymerase chain reaction
PI Plaque index
PIBI Periodontal inflammatory burden index
PISF Peri-implant sulcular fluid
PMN Polymorphonuclear neutrophil
POC Point-of-care
PPD Probing pocket depth
qPCR Quantitative polymerase chain reaction
ROC Receiver operating characteristics
sCD40L Soluble CD40 ligand
SD Standard deviation
SDD Sub-antimicrobial dose
SDS Sodium dodecyl sulfate
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMC Smooth muscle cell
STEMI ST-elevation myocardial infarction
Td-CTLP T. denticola chymotrypsin-like proteinase, dentilisin
TIA Transient ischemic attack
TIMP Tissue inhibitor of matrix metalloproteinase
TN True negative
TNF-α Tumor necrosis factor α
TnT Troponin T
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>TP</td>
<td>True positive</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type-2 diabetes mellitus</td>
</tr>
<tr>
<td>UAP</td>
<td>Unstable angina pectoris</td>
</tr>
<tr>
<td>VLDL-IDL</td>
<td>Very low-density lipoprotein-intermediate-density lipoprotein</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
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1 INTRODUCTION

Periodontitis and atherosclerotic cardiovascular diseases (CVDs) are common chronic inflammatory diseases worldwide. Atherosclerotic CVDs result in approximately 30% of deaths globally, representing the leading cause of death (Lockhart et al. 2012).

Traditionally, inflammation was characterized by the following cardinal signs, defined in the first century AD by the Roman academic Celsus: redness (rubor), swelling (tumor), heat (calor), pain (dolor), and a disturbance of function (functio laesa). This inflammatory reaction is the body’s attempt to restrict the pathogenic stimuli, and an imbalance in the inflammatory process leads to the development of disease. If the host’s immune response is insufficient to exceed and clear the challenge, the acute inflammation may become chronic (Kurgan & Kantarci 2018). Periodontitis and atherosclerotic CVDs serve as examples of chronic inflammatory diseases, where the degradation of tissue, particularly the extracellular matrix (ECM), is crucial.

Periodontitis and CVD share similarities in pathophysiology as well as associate with one another. Periodontitis is an independent risk factor for CVD (Lockhart et al. 2012), and the proatherogenic effects of periodontitis include an increased systemic low-grade inflammation, bacteremia, endotoxemia, and molecular mimicry (Schenkein & Loos 2013). Periodontitis and CVD share common risk factors and possibly some genetic background (Genco et al. 2013, Laine et al. 2012). Additionally, genetic variation may explain as much as 40% of the variation in levels of systemic inflammatory biomarkers (Schnabel et al. 2009). Furthermore, smoking represents one of the most well-known risk factors for periodontitis and CVD, and leads to several adverse effects on systemic and oral health (Genco et al. 2013).

Since periodontitis and atherosclerotic CVD burden humans globally, and periodontitis is associated with an increased CVD risk, biomarkers for diagnosing and predicting periodontitis and CVD remain important to investigate. Such biomarkers could predict the transition of gingivitis to active periodontitis or the shift from stable atherosclerotic plaque to unstable, vulnerable plaque. The problem with using a single biomarker lies in the pivotal role of the molecules involved in inflammation, their contribution to both the initiation of periodontal and atherosclerotic disease as well as to the healing process, thus hindering the predictive value of the future course of disease. Identifying additional markers in combination or as a panel would reflect the different phases of the diseases and could improve the predictive
abilities. The beauty of a predictive biomarker or biomarker combination lies in the potential to reveal more about the phenomenon behind clinical signs and symptoms. Namely, this would allow clinicians to add some crucial information beyond clinical parameters and aid the development of personalized treatment strategies.

Matrix metalloproteinases (MMPs) are important proteases in many normal and pathological conditions in humans (Iyer et al. 2012). In humans, 23 different MMPs have been characterized (Jackson et al. 2010). Furthermore, MMPs are primarily inhibited by their endogenous inhibitor, the tissue inhibitor of matrix metalloproteinase (TIMP-1) (Brew et al. 2000). MMP-8 and MMP-9 remain important ECMs degrading enzymes both in periodontitis and atherosclerotic CVD, while TIMP-1 reflects the balance between tissue destruction and inhibition (Rathnayake et al. 2015, Johnson et al. 2017). If the amount of MMP-8 and MMP-9 exceeds a physiological threshold, the tissue damage change becomes irreversible resulting in continuous and unmanageable inflammation. These enzymes can be regarded as biomarkers of periodontitis when measured from the saliva and for CVD when measured from circulation. Furthermore, smoking compromises the functioning of the immune system, thereby also affecting local and systemic biomarkers (Tymkiw et al. 2011). Finally, several other factors may influence these biomarkers, rendering their investigation important.
2 REVIEW OF THE LITERATURE

2.1 ATHEROSCLEROSIS

Arterial wall
The structural integrity of the arterial wall is maintained by the extracellular matrix (ECM) (Figure 1). Type I and III collagen are prominent structures in the arterial wall, and the artery wall includes macrophages and smooth muscle cells (SMCs) (Papazafiropoulou et al. 2009). Type I collagen provides rigidity and tensile strength, whereas type III collagen provides elasticity with its weave-like pattern. In pathological CVD conditions such as hypertension, an altered collagen composition including an increase in the type I/III collagen ratio is detected (Jalil et al. 1989).

Figure 1. The structure of the arterial wall. Modified from Yurdagul et al. 2016.
Inflammation plays a crucial role in atherosclerotic CVDs together with pathological lipid accumulation under the artery intima. The etiology behind atherosclerosis and CVD is multifactorial. For instance, risk factors include dyslipidemia, high blood pressure, smoking, diabetes, obesity, a sedentary lifestyle, an unhealthy diet (that is, a diet containing lots of saturated and trans fatty acids, cholesterol, sodium, and sugar), age, sex, alcohol consumption, and genetics. Atherosclerosis can lead to atherosclerotic plaque rupture, hemorrhage, the formation of thrombus, and an acute CVD event (Falk et al. 1995, Shah 1998).

Histopathologically vulnerable plaques (Figure 2) present the following features: a large and lipid-rich atheromatous core, a thin fibrous cap, and active infiltration of inflammatory cells, especially macrophages (Weissberg 1999). In addition, a decreased SMC density, the proliferation of SMCs (Ravn & Falk 1999), a reduced amount of collagen in the fibrous cap, and an increased vascularature in the plaque have been observed (Shah 1998). Tissue-degrading enzymes, such as MMP-9 (Kalela et al. 2002) and MMP-8 (Molloy et al. 2004), participate in the rupture process. The plaque vulnerability depends on the size of the lipid-rich core, the cap thickness, and inflammation as well as the functioning of the repair processes (Ravn & Falk 1999).

Several differences distinguish vulnerable from stable plaques. Vulnerable plaques exhibit less collagen and SMCs in the cap and shoulder regions and more inflammatory cells compared to stable plaques (Pasterkamp et al. 1998). Paradoxically, this aggressive remodeling in the plaque prevents expansive enlargement and luminal narrowing, but predisposes atherosclerotic plaque to rupture and exhibit clinical manifestations (Pasterkamp et al. 2000).
Clinical manifestations of advanced cardiovascular disease (CVD)

Acute coronary syndrome
Acute coronary syndrome (ACS), including acute myocardial infarction (AMI) and unstable angina pectoris (UAP), is a syndrome in which the blood supply to the myocardium is blocked. AMI is an acute clinical manifestation of atherosclerotic plaque rupture followed by myocardial ischemia and irreversible myocardial tissue injury and ischemia (Yabluchanskiy et al. 2013). In comparison, UAP is caused by a sudden narrowing of the vascular lumen leading to stenosis of the artery and a limited blood supply to the cardiac muscle.

Ischemic stroke
Ischemic stroke can result from atherosclerotic plaque rupture and thrombus formation in the brain artery or thrombus dissemination blockig the brain vessels. Atrial fibrillation also predisposes individuals to ischemic stroke. Transient ischemic attack (TIA) is a reversible dysfunction of the blood flow to the brain or the retina without signs of infarction or permanent damage. The etiology of ischemic and hemorrhagic stroke differs. However, hemorrhagic...
transformation (HT) may naturally develop following cardiogenic ischemic stroke (Toni et al. 1996). The severity and the impairment caused by stroke can be objectively quantified using the National Institutes of Health Stroke Scale (NIHSS), which measures stroke severity on a scale from 0 to 42 (Brott et al. 1989).

2.2 PERIODONTITIS

Periodontitis is a chronic and irreversible inflammatory disease in tooth-supporting tissues, periodontium, caused by a polymicrobial infection (Darveau 2010, Kinane et al. 2017) (Figure 3). A pathogenic bacterial challenge and biofilm dysbiosis lead to the activation of a host response. That process results in the migration of polymorphonuclear (PMN) cells and the degranulation of tissue-degrading enzymes, such as MMP-8 and MMP-9, along the inflamed periodontal tissue (Sorsa et al. 2006, Uitto et al. 1990). Adaptive immunity cells, Th1 lymphocytes, are also involved in periodontitis, which appear to play an important role during the initiation of chronic periodontitis, whereas Th2 lymphocytes might be more important during later stages (Gemmell et al. 2004).

While gingivitis precedes periodontitis, it remains unclear at which point gingivitis transforms into periodontitis (Kurgan & Kantarci 2018). Bleeding on probing (BOP) represents the primary parameter for diagnosing gingivitis (Caton et al. 2018). Gingivitis is reversible, although under specific conditions disease in susceptible individuals advances into irreversible chronic periodontitis (Kinane et al. 2017). Periodontitis is characterized as gingival bleeding, connective tissue breakdown, and alveolar bone loss (ABL). The inflamed dentogingival surface area ranges from 8 cm² to 20 cm² in periodontitis patients (Hujoel et al. 2001). In addition, the severity of periodontitis is affected by modifiable and nonmodifiable environmental and host risk factors (Kinane et al. 2017). Previously, periodontitis was classified into four different types: chronic periodontitis, aggressive periodontitis, necrotizing periodontitis, and periodontitis as a manifestation of systemic disease, although new classification criteria for periodontal diseases recently appeared (Tonetti et al. 2018). Currently, characterizing periodontitis relies on the clinical attachment loss (CAL), the form of periodontitis (that is, periodontitis, necrotizing periodontitis, or periodontitis as a manifestation of systemic disease), and the progression rate of periodontal disease (stage and grade) (Tonetti et al. 2018).

Periodontitis associates with several risk factors, such as smoking, diabetes, age, sex, metabolic syndrome, osteoporosis, alcohol consumption,
socioeconomic status, ethnicity, stress, and genetic factors (Genco et al. 2013). The relationship between periodontitis and diabetes is bidirectional: that is, diabetes is a distinct risk factor for periodontitis; untreated periodontitis may adversely affect glycemic control in diabetic patients (Lalla et al. 2011). Furthermore, a few studies found that periodontitis or advanced tooth loss associate with an increased risk for incident diabetes (Winning et al. 2017a, Winning et al. 2017b; Liljestrand et al. 2015). Additionally, obese subjects often exhibit elevated levels of proinflammatory cytokines causing a systemic low-grade inflammation, which can predispose an individual to periodontitis, although a causal relationship between obesity and periodontitis remains lacking (Shungin et al. 2015).

![Inflammatory processes and clinical signs of periodontitis](image)

**Figure 3.** Inflammatory processes and clinical signs of periodontitis. Modified from Trindade et al. 2014. APC= antigen-presenting cell, NK cell= natural killer cell, PMN= polymorphonuclear neutrophil, MMP= matrix metalloproteinase, LPS= lipopolysaccharide.

Although periodontitis is an inflammatory reaction to a polymicrobial biofilm, several individual species such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Prevotella intermedia* have been associated with periodontitis (Haffajee et al. 1998). In addition, these individual species can be used as bacterial biomarkers for periodontitis. These selected bacteria possess community-wide effects known as “keystone
pathogens” and appear crucial for the development of dysbiosis (Hajishengallis et al. 2014). Clinical measures of periodontitis include the measurement of the probing pocket depth (PPD), BOP, CAL, and ABL evaluated from radiographs (Armitage 2004). These clinical measures can be supplemented with microbial and enzymatic tests.

2.3 SMOKING AS A RISK FACTOR FOR PERIODONTITIS AND CVD

Smoking represents a well-known risk factor for pulmonary diseases, CVDs, periodontitis, various cancers, and all-cause mortality. Reviews have demonstrated that tobacco smoking carries the highest attributable disability-adjusted life years (DALYs) (170.9 million DALYs) when taking into account alcohol, tobacco, and illicit drug use globally (Peacock et al. 2018). The prevalence of daily tobacco smoking has been estimated at 15.2% in the adult population worldwide (Peacock et al. 2018). Cigarette smoke includes more than 4 000 different toxins, such as nicotine, carbon monoxide, oxidizing radicals, and carcinogens (Genco et al. 2013). In addition, smoking can be characterized along three dimensions: the intensity, duration, and time since cessation (Liu et al. 2016). Finally, genetics strongly affect smoking and nicotine dependence. Genetic factors may either influence the central nervous system or nicotine intake and metabolism (Loukola et al. 2014). The genetic variation in chromosome 15 has been associated with smoking-related phenotypes (Loukola et al. 2014).

**Systemic effects of smoking**

Smoking causes systemic oxidative stress and inflammation. For example, the C-reactive protein (CRP) is significantly elevated in smokers compared to nonsmokers (Yanbaeva et al. 2007) (Figure 4). Many smoking-related changes are reversible after quitting, but CRP remains permanently elevated even in former smokers (Yanbaeva et al. 2007).

Smoking has been found to dose-dependently increase the total white blood cell (WBC) count (Yarnell et al. 1987), whereas another study demonstrated that current smoking further increased the total WBC count, particularly the granulocytes, more than cumulative exposure calculated as pack years of smoking (Smith et al. 2003). Smoking also decreases the concentrations of several cytokines and chemokines, thus causing immunosuppressant effects and an enhanced susceptibility to periodontitis and CVD (Tymkiw et al. 2011).
Review of the literature

Figure 4. Possible mechanisms of smoking affecting CVD and periodontal parameters. Modified from Chalouhi et al. 2012. CVD= cardiovascular disease, CRP= C-reactive protein

**Smoking and periodontitis**

Smoking increases the risk for periodontitis approximately two to six times (Heasman et al. 2006). Interestingly, the risk for periodontitis remains elevated in former and involuntary smokers (Heasman et al. 2006, Nishida et al. 2008), while the risk for periodontitis decreases as a function of time after smoking cessation (Warnakulasuriya et al. 2010).

In addition, smoking impairs wound healing and accelerates the breakdown of periodontal tissues, thereby disturbing the normal host response (Giuca et al. 2014) (Figure 4). Nicotine can stimulate neutrophils in a more sensitive direction against periodontal bacterial challenges by impairing the neutrophil functioning and enhancing degranulation (Söder et al. 2002). Nicotine also inhibits the proliferation of periodontal ligament fibroblasts (Giannopoulou et al. 1999). Furthermore, smoking hinders PMN activity in peripheral blood PMN leukocytes in a dose-dependent manner (Zappacosta et al. 2000).
Smoking can also elevate the amount of salivary fluid (Giua et al. 2014), and either increase (Ustün et al. 2007) or decrease the gingival crevicular fluid (GCF) volume (Morozumi et al. 2004). Smoking reduces the oxygen supply in periodontal tissues by causing vasoconstriction (Chen et al. 2001, Scott et al. 2004, Hayman et al. 2011). Thus less gingival bleeding is clinically observed and the disease severity remains partially masked (Hayman et al. 2011). Periodontitis appears as a specific and complicated manifestation in smokers characterized as fibrotic gingiva, less gingival bleeding (Chen et al. 2001), deeper gingival pockets, more gingival recesions, and no association between oral hygiene and periodontal status when compared to never smokers (Heasman et al. 2006). The effect of periodontal treatment diminishes in smokers compared to nonsmokers (Koss et al. 2016), but smoking cessation improves the responsesiveness to periodontal therapy (Preshaw et al. 2005). Recovery of periodontal microcirculation after smoking cessation is a crucial factor contributing to improvements to periodontal health (Morozumi et al. 2004).

Smokers exhibit deeper pockets and more attachment loss than nonsmokers (Torrungruang et al. 2012, Gupta et al. 2016), and a dose-dependent effect has been observed based on attachment loss (Grossi et al. 1994) and ABL (Grossi et al. 1995). Smoking even associates with tooth loss despite patient sex or ethnic background (Arora et al. 2010, Ahlqwist et al. 1989, Krall et al. 1997, Dietrich et al. 2007, Albandar et al. 2000, Krall et al. 2006, Chen et al. 2001, Warnakulasuriya et al. 2010). Finally, smoking is also a risk factor for implant failure (Warnakulasuriya et al. 2010).

2.4 THE ASSOCIATION BETWEEN PERIODONTITIS AND CVD

Inflammation is a crucial factor in the atherosclerotic process (Libby 2002, Ridker et al. 2017). Several studies identified the association between chronic infections and CVD (Kiechl et al. 2001, Espinola-Klein et al. 2002, Kholy et al. 2015). Chronic oral infections have been investigated as a risk factor for CVD for decades. In fact, poor oral health associates with coronary heart disease (CHD) and AMI (Mattila et al. 1989, DeStefano et al. 1993, Beck et al. 1996). In addition, the American Heart Association identified periodontitis as an independent risk factor for CVD in 2012 (Lockhart et al. 2012). Moreover, periodontitis and CVD share several common risk factors. Periodontal treatment reduces systemic inflammation and improves endothelial functioning, thus supporting the association between periodontitis and CVD (Tonetti and Van Dyke 2013, Tonetti et al. 2007, Teeuw et al. 2014). However,
while periodontitis and CVD are clearly associated with each other, the causal relationship between these two diseases remains unclear.

Statins are frequently used as medications for hypercholesterolemia, but they may also affect periodontium. Statins inhibit intrinsic cholesterol production in the liver by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase in the mevalonate pathway. In addition, statins have many pleiotropic effects, such as decreasing inflammation, reducing monocyte adhesion to the endothelium (Weber et al. 1999), decreasing macrophage infiltrates (Shiomi et al. 1995, Bustos et al. 1998), and promoting repair (Weissberg 1999). Statins can also inhibit several MMPs, from both SMCs and macrophages, possibly contributing to the plaque-stabilizing effects of statins (Luan et al. 2003). Statins also appear to affect periodontal parameters. More specifically, patients undergoing statin therapy exhibited fewer signs of periodontitis, such as a lower number of pathological periodontal pockets, a smaller periodontal inflammatory burden index (PIBI), and a reduced tooth loss rate, when compared to patients not receiving statins (Lindy et al. 2008, Cunha-Cruz et al. 2006).

Chronic oral infections have been associated with cerebral infarction (Syrjänen et al. 1989, Grau et al. 1997, Palm et al. 2016). Gingivitis, periodontitis, and edentulosity have been associated with an increased risk for stroke, nonhemorrhagic (Wu et al. 2000) and ischemic stroke in particular caused by large-artery atherosclerosis, cardioembolism, and cryptogenic etiology (Grau et al. 2004, Elter et al. 2003). Deepened gingival pockets, attachment loss (AL), BOP, the plaque index (PI), and elevated levels of *P. gingivalis* as signs of periodontitis all associated with ischemic or hemorrhagic stroke (Ghizoni et al. 2012). Serum IgA antibody levels against *A. actinomycetemcomitans* and *P. gingivalis* also associated with an increased risk for incident stroke, whereas increased IgA antibody levels against *P. gingivalis* associated with the risk for recurrent stroke (Pussinen et al. 2004; Pussinen et al. 2007). Interestingly, *A. actinomycetemcomitans* leukotoxin-neutralizing antibodies, which may carry a protective effect against CVD by preventing macrophage damage, associated with a decreased risk for stroke in women (Johansson et al. 2005).

Periodontitis may associate with CVD and atherosclerosis through direct and indirect mechanisms involving several pathways. Over 700 bacterial species have been found in the oral cavity, approximately 400 of which originate from periodontal pockets (Benn et al. 2018, Lockhart et al. 2008). Periodontal pathogens, such as *A. actinomycetemcomitans*, *T. forsythia*, *P. gingivalis*, and *P. intermedia*, may enter circulation causing bacteremia due to daily routines such as chewing and tooth brushing. These species originating in the
oral cavity have been found in the atherosclerotic plaques of the artery wall (Haraszthy et al. 2000). Additionally, periodontitis can lead to endotoxemia, characterized as elevated levels of systemic lipopolysaccharide (LPS), an endotoxin. LPS is a membrane structure of gram-negative bacteria associated with atherosclerosis, CVD, and metabolic diseases (Piya et al. 2013, Kallio et al. 2015). Moreover, LPS activity associates with pro-atherogenic lipoprotein classes, namely a very low-density lipoprotein–intermediate-density lipoprotein (VLDL–IDL) fraction (Kallio et al. 2008), thereby contributing to foam-cell formation.

The direct mechanisms believed to connect periodontitis to CVD include bacteremia and vascular inflammation caused by periodontal pathogens. In comparison, the indirect mechanisms include elevation of systemic inflammatory markers, an immunological response due to periodontitis (Noack et al. 2001), and immune cross-reactivity, that is, molecular mimicry. These systemic pro-inflammatory mediators may originate directly from periodontal lesions or may be systemically produced (Schenkein & Loos 2013). Periodontal inflammation can affect the liver, whereas increased thrombotic and hemostatic markers as well as dyslipidemia can result from periodontitis (Schenkein & Loos 2013). Furthermore, periodontitis can stimulate innate and adaptive immunity, and auto-reactive Th cells, whereby cross-reactive auto-antibodies are generated due to molecular mimicry (Schenkein & Loos 2013). At an advanced stage of atherosclerosis, periodontal inflammation can promote plaque rupture and thrombus formation by affecting SMC migration, thrombin generation, the promotion of a Th1-type response, and accelerating collagen degradation (Schenkein & Loos 2013). Moreover, a hypothetical link between periodontitis and CVD may be found from the proteases produced by periopathogens (such as \textit{P. gingivalis} originated gingipains) and their stimulation of latent host MMPs in a more active direction (Imamura et al. 2003). Additionally, \textit{P. gingivalis} can induce dysbiosis in the gut microbiota and promote endotoxemia. This \textit{P. gingivalis}-induced gut dysbiosis represents a novel mechanism potentially associating periodontitis with systemic diseases (Arimatsu et al. 2014).

Periodontitis and CVD may also share common genetic biomarkers given that both diseases are complex and typically polygenic (Laine et al. 2012). The effect of environmental factors for both diseases remains crucial. Genome-wide association studies (GWAS) have identified several candidate loci associating with chronic (Divaris et al. 2013) and aggressive periodontitis (Schaefer et al. 2010). Furthermore, GWAS found several loci associated with CVD, whereby the most notable for associations with coronary artery disease (CAD) and myocardial infarction (MI) were observed at chromosome position 9q21.3 (Consortium, W.T.C.C 2007, Helgadottir et al. 2007, Schunkert et al.
Review of the literature

2008). The most-studied locus situated in that position is ANRIL, which associates with CVD (Congrains et al. 2013) and periodontitis (Schaefer et al. 2009, Aarabi et al. 2017), particularly its aggressive form (Schaefer et al. 2011). Finally, epigenetics, investigating heritable changes in the gene function without a change in the DNA sequence, may provide new information about the link between genetics, disease, and environmental factors (Schulz et al. 2016). For example, smoking exposure significantly and epigenetically affects DNA methylation on leukocytes, such as monocytes, granulocytes, and B and T cells, (Su et al. 2016). These alterations could modulate both periodontitis and CVD.

2.5 ENZYMES IN TISSUE DESSTRUCTION

2.5.1 MATRIX METALLOPROTEINASES (MMPS)

Matrix metalloproteinases (MMPs) play an important role in several normal physiological processes, such as embryonic development, tissue morphogenesis, and wound repair, as well as in pathological conditions, such as CVDs, periodontitis, inflammatory diseases, and cancer (Sternlicht & Werb 2001, Nelson et al. 2000, Nagase & Woessner 1999). In humans, 23 different MMPs have been identified (Jackson et al. 2010). MMPs can be divided into collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysin (MMP-7), macrophage metalloelastases (MMP-12), and membrane-type MMPs (MMP-14, MMP-17, MMP-25, and MMP-26) (Johnson 2017).

MMPs are synthesized as preproenzymes and secreted as inactive proenzymes (pro-MMPs) (Nagase & Woessner 1999). These latent pro-MMPs contain a cysteine switch, an intramolecular complex between the single cysteine residues situated in the propeptide domain. This cysteine switch ligates the catalytic zinc, thereby maintaining the enzyme as inactive (Nagase & Woessner 1999, Van Wart & Birkedal-Hansen 1990). The activation of MMP requires the dissociation of the cysteine switch from the active site of MMP (zinc and 2-3 calcium ions) (Van Wart & Birkedal-Hansen 1990). Because of various functions in the human body, MMPs are tightly regulated at different levels, namely, the transcriptional, post-transcriptional, and protein levels (Sternlicht & Werb 2001).

Many MMPs are found in the gingival crevicular fluid (GCF), but MMP-8 accounts for approximately 80% of the total collagenase protein found in GCF (Golub et al. 2008). The other most widely found MMPs in GCF consist of MMP-9, MMP-13, and MMP-1 (in that order) in chronic periodontitis patients.
(Golub et al. 2008, Choi et al. 2004). Additionally, MMP-2, MMP-3, MMP-7, and MMP-14 have been linked to periodontitis (Tuter et al. 2005, Tervahartiala et al. 2000, Beklen et al. 2006).

In atherosclerotic plaque endothelial cells, vascular smooth muscle cells (VSMCs) and macrophages can secrete different MMPs. Endothelial cells can serve as the source of MMP-1, MMP-3, MMP-8, MMP-9, MMP-10, and MMP-11; VSMCs serve as the source for MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-11, MMP-14, and MMP-16; and macrophages serve as the source for MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, and MMP-16 (Johnson 2017).

### 2.5.2 MMP-8 (HUMAN NEUTROPHIL COLLAGENASE, COLLAGENASE-2)

The molecular weight of latent pro-MMP-8 is 85 kDa, which drops to 65 kDa following proteolytic activation (Knäuper et al. 1990). The gene for MMP-8 is situated at 11q22.3 (Pendás et al. 1996). When compared to other MMPs, MMP-8 is the most potent enzyme in the initiation of the degradation of type I collagen, the main substrate of MMP-8 (Sorsa et al. 2006). Additionally, MMP-8 can degrade type II and III collagen, as well as fibrinogen, laminin, and pro-MMP-8 (Hiller et al. 2000, Pirilä et al. 2003, Knäuper et al. 1993). MMP-8 is released from granules of neutrophils following an inflammatory stimulus (Sternlicht & Werb 2001). MMP-8 in atherosclerotic plaques are primarily secreted by the macrophages (Bäck et al. 2010). Interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α) act as inhibitors and regulators of MMP-8 (Bäck et al. 2010). MMP-8 is primarily regulated by affecting the secretion or inhibition, while neutrophil-origin MMP-8 is not regulated much at the transcription level (Wang et al. 2004, Jeong et al. 2015, Ogut et al. 2016).

### 2.5.3 MMP-9 (GELATINASE B, 92-KDA TYPE IV COLLAGENASE)

MMP-9 is a 92-kDa enzyme in the proform and 88-kDa enzyme in the active form in humans (Papazafiropoulou et al. 2009). The MMP-9 chromosome location lies at 20q11.2–q13.1 (Bäck et al. 2010). MMP-9 primarily degrades type IV collagen, the main component of the basement membrane (Ye 2000) (Figure 1). The catalytic domain of gelatinases includes three repeats of the fibronectin-type II domain, which interacts with collagens and gelatins (Steffensen et al. 1995). Macrophages, especially foam cells, secrete MMP-9 predominantly in the shoulder region of atherosclerotic plaque (Yabluchanskiy et al. 2013, Bäck et al. 2010). Additionally, MMP-9 expression is common in leukocytes and their precursors (Bäck et al. 2010). Furthermore, MMP-9 expression is primarily regulated at the transcription level, and several
cis elements in the promoter region play important roles in this regulation process (Zhang et al. 1999). The release of MMP-9 from neutrophil granules occurs after the activation of neutrophils (Sternlicht & Werb 2001). Finally, oxidative stress can activate MMP-9 (Siwik et al. 2001).

2.5.4 THE REGULATION OF MMPS

Activation of MMPs
The activation of MMPs can occur in various ways, such as due to other proteases (trypsin and MMP autolysis), sodium dodecyl sulfate (SDS), heavy metals or oxidatively (such as via NaOCl)(Van Wart & Birkedal-Hansen 1990). In addition, the regulation of MMP activity can occur through the regulation of MMP gene expression (Auble et al. 1991), through the regulation of MMP activity via the cysteine switch mechanism (Lijnen et al. 1999), or through the inhibition of MMPs by TIMPs. Reactive oxygen species can activate latent MMPs via the cysteine switch mechanism, that is, by reacting with thiol group MMPs (Rajagopalan et al. 1996, Van Wart & Birkedal-Hansen 1990). Without additional exogenous stimulation, macrophage foam cells from the atheroma produce reactive oxygen species which can modulate MMP-2 and MMP-9 activities (Rajagopalan et al. 1996). A chronic increase in the blood flow can upregulate and activate MMP-2 and MMP-9 in the arterial wall via nitric oxide (NO) (Tronc et al. 2000).

Bacterial proteases from periodontal pathogens, such as gingipains produced by P. gingivalis, can both stimulate MMP expression and activate latent MMPs (Imamura et al. 2003). Gingipains are trypsin-like cysteine proteinases and very potent virulence factors (Imamura et al. 2003). Another example whereby the host MMP activating bacterial proteases is T. denticola chymotrypsin-like proteinase (Td-CTLP, dentilisin), found to activate latent MMP-8 and MMP-9 in orodigestive tumors (Niemin et al. 2018). Additionally, Td-CTLP can degrade TIMP-1, thus also affecting a tissue-destructive cascade (Niemin et al. 2018). A. actinomycetemcomitans and its virulence factor leukotoxin can stimulate secretion and the extracellular activation of MMP-8 by binding to the neutrophil surface receptor. This initiates an intracellular signal cascade leading to degranulation in human neutrophils (Claesson et al. 2002). In addition, LPS from P. gingivalis can modulate the expression of MMP and TIMP from human gingival fibroblasts (HGFs) (Bozkurt et al. 2017).

Proteolytic activation decreases the molecular weight, whereas chemical activation can occur via the disulfide exchange mechanism without a change in the molecular weight (Van Wart & Birkedal-Hansen 1990). In vitro MMP-
8 and MMP-9 can be activated chemically by 4-aminophenylmercuric acetate (APMA) (Grierson et al. 2010).

**Inhibition of MMPs**

MMPs are primarily inhibited by their endogenous inhibitor, the tissue inhibitor of metalloproteinase (TIMP) (Van Wart & Birkedal-Hansen 1990, Brew et al. 2000). TIMPs inhibit MMPs by affecting catalytic zinc via the N-terminal cysteine residue and protein–protein surface interactions (Visse et al. 2003, Gomis-Ruth et al. 1997). TIMP-1 can also regulate the proliferation of fibroblasts and influence collagen production (Lovelock et al. 2005). In addition, TIMP-1 appears to regulate apoptosis and angiogenesis (Ries 2014). In circulation, α2 macroglobulin inhibits MMPs, thus preventing systemic MMP activation (Yabluchanskiy et al. 2013).

MMPs can also be inhibited by several other strategies besides TIMP, most commonly by targeting active-site zinc (Levin et al. 2017). Antibody-based (active site antibody or allosteric antibody), small molecule inhibitors, the endogenous-like protein inhibitors, and prodomain-based MMP inhibitors have all been investigated (Levin et al. 2017). Additionally, some drugs, such as a subantimicrobial dose (SDD) doxycycline (Golub et al. 1995, Tronc et al. 2000, Bendeck et al. 2002) and bisphosphonates (Teronen et al. 1999), can inhibit MMPs. In clinical use many obstacles affect MMP inhibition because of the lack of specificity and its broad inhibitory profile. Cross-reactivity between different MMPs and the developed inhibitors are of great interest when targeting MMPs in the human body.

**2.5.5 THE MMP/TIMP-1 RATIO**

TIMP-1 interacts with MMP-8 and MMP-9 at a 1:1 stoichiometry and a high affinity (Nagase & Brew et al. 2003, Van Wart & Birkedal-Hansen 1990). The formation of the MMP–TIMP complex depends on the hemopexin-like domain (Bode 1995). The MMP/TIMP ratio potentially describes “the net MMP activity” or overall proteolytic potential of MMPs, that is, the degenerative ECM capacity (Bonnema et al. 2007). The balance between MMPs and TIMPs is crucial in many pathological conditions, such as periodontitis, CVD, chronic inflammatory states, and cancer (Farr et al. 1999).
2.5.6 MPO

Myeloperoxidase (MPO), or the “green heme protein”, can be regarded as a biomarker of oxidative stress and inflammation. MPO is a lysosomal enzyme primarily produced by PMN neutrophils (Khan et al. 2018) during the early stages of PMN proliferation (van Eeden et al. 2000) and by monocytes (Podrez et al. 2000). The molecular weight of the active enzyme is 150 kDa, and the gene for MPO is located at chromosome 17 (Khan et al. 2018). MPO is stored in different neutrophil granules than MMP-8 (Borregaard et al. 2007). Oxidative stress and inflammatory mediators can cause MPO release into the extracellular fluid, and the antibacterial effect of MPO results from the secretion of H$_2$O$_2$ (Khan et al. 2018). MPO can oxidatively activate pro-MMP-8 and pro-MMP-9 and deactivate TIMP-1 (Saari et al. 1990) (Figure 5). MPO accelerates atherosclerosis through its involvement in the oxidation of low-density lipoprotein (LDL), contributing to endothelial dysfunction (Tay et al. 2015). Furthermore, MPO carries the unique ability to generate a powerful oxidant in physiological conditions using the following equation:

$$\text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \xrightarrow{\text{MPO}} \text{HOCl} + \text{H}_2\text{O}$$

Figure 5. The function of MMP-8, MMP-9, TIMP-1, and MPO in collagen and gelating degradation. Modified from Libby 2008.
2.6 BIOMARKERS

Biomarkers quantitatively characterize biological signs and are reproducible. Biomarkers are used for the characterization of normal biological or pathogenic processes, as well as for pharmagolocical responses (Biomarkers Definitions Working Group 2001). Any biomarker should be objectively measured and evaluated. Thus, a biomarker should fulfill the following criteria: a) accuracy, b) reliability, and c) therapeutic impact and usefulness for early intervention (Redberg et al. 2003). The advantages of biomarkers lie in their prediction ability and their application for developing personalized medicine and dentistry. Specifically, biomarkers that predict outcomes and identify high-risk patients would be useful to develop. Biomarkers should be validated, and discussions persist regarding when a biomarker is sufficient for clinical use (Lin et al. 2009). When biomarkers are used as diagnostic tests the sensitivity and specificity should be evaluated.

<table>
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<tr>
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<th>Disease</th>
<th>No disease</th>
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<tr>
<td><strong>Test positive</strong></td>
<td>True positive</td>
<td>False positive</td>
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<tr>
<td><strong>Test negative</strong></td>
<td>False negative</td>
<td>True negative</td>
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The sensitivity (“real positives”) describes the probability of a biomarker existing in which a diseased patient is diagnosed as diseased.

\[
\text{sensitivity} = \frac{\text{true positives}}{(\text{true positives} + false negatives)}
\]

The specificity (“real negatives”) describes the probability of a biomarker existing in which a healthy person is diagnosed as healthy.

\[
\text{specificity} = \frac{\text{true negatives}}{(\text{true negatives} + false positives)}
\]

**Samples**

**Blood fluids: serum and plasma**

The concentrations of MMPs and their endogenous inhibitors vary depending on the sample material. MMP-8 measured from serum exhibited higher concentrations compared to plasma samples, while serum with clot activators presented higher MMP-8 concentrations compared to serum without clot
activation (Jung et al. 2008). The release of MMP-8 may occur during coagulation, possibly explaining such differences (Jung et al. 2008, Salminen et al. 2017). The concentrations of MMP-9 and TIMP-1 were highest when measured from serum compared with ethylenediaminetetraacetic acid (EDTA) plasma or heparin plasma, whereby heparin plasma samples presented the lowest concentrations (Jung et al. 1996, Jung et al. 1998).

**Oral fluids**

Oral fluids, such as saliva, GCF, peri-implant sulcular fluid (PISF), and mouth rinse, contain serum proteins, host tissue products, inflammatory mediators, enzymes, and microbial metabolites (Buduneli et al. 2011, Sorsa et al. 2011). Whole saliva contains GCF, electrolytes, serum, epithelial cells, immune cells, microorganisms, and bronchial products (Chojnowska et al. 2018, Miller et al. 2006). Saliva reflects oral diseases, potentially also reflecting the systemic status (Miller et al. 2006, Chojnowska et al. 2018).

Several advantages exist related to the use of saliva for diagnostic purposes. Saliva is easy to collect noninvasively and inexpensively, and is safe to administer (Javaid et al. 2016). The collection of saliva can also be performed at home without necessitating trained medical staff (Javaid et al. 2016).

### 2.6.1 MMP-8 AS A BIOMARKER IN CVD AND PERIODONTITIS

**MMP-8 in atherosclerosis and CVD**

MMP-8 has been found in the shoulder regions of rupture-prone atherosclerotic plaques, confirming the role of MMP-8 in atherogenesis (Herman et al. 2001, Peeters et al. 2011). Active MMP-8 was elevated in the atherosclerotic plaques of symptomatic patients (samples collected from patients undergoing carotid endarterectomy or CEA), and these plaques more often showed signs of intraplaque hemorrhage (Molloy et al. 2004). Serum MMP-8 and the MMP-8/TIMP-1 molar ratio were significantly elevated in men with subclinical atherosclerosis compared to those without (Tuomainen et al. 2007). In addition, high concentrations of MMP-8 were also detected in aneurysm and rupture site biopsies (Wilson et al. 2005, Wilson et al. 2006).

Elevated plasma MMP-8 associated both with the presence and severity of CAD, presenting a stepwise increase in MMP-8 concentrations according to the number of stenotic vessels (Kato et al. 2005). As such, patients with arterial disease (symptomatic aorto-occlusive disease, carotid artery stenosis, or an abdominal aortic aneurysm) exhibited elevated serum MMP-8 and a
MMP-8/TIMP-1 ratio compared to the reference group (Pradhan-Palikhe et al. 2010).

Moreover, ACS and stable CAD patients had significantly elevated serum and plasma MMP-8 levels compared to healthy controls (Pussinen et al. 2013, Momiyama et al. 2010), while the concentrations of serum MMP-8 in ACS patients decreased significantly during follow-up (Pussinen et al. 2013). Serum MMP-8 levels were lower in ST-elevation myocardial infarction (STEMI) patients compared to non-STEMI patients (Pussinen et al. 2013). While elevated systemic MMP-8 concentrations have been observed, lower total saliva MMP-8 levels have also been measured in AMI patients compared to non-AMI patients (Buduneli et al. 2011).

Plasma MMP-8 did not differ between stroke, TIA, and asymptomatic patients, but those patients with histological features of vulnerable plaques exhibited higher MMP-8 levels compared to patients with stable plaques (Turu et al. 2006). Additionally, a study exists in which lower systemic MMP-8 values were measured in stroke patients compared to controls (Lorenzl et al. 2003). However, in a recent study an elevated serum MMP-8 level significantly associated with acute ischemic stroke and stroke severity (Palm et al. 2018).

Several studies have also investigated the MMP-8 ratio with its endogenous inhibitor TIMP-1. As such, the serum MMP-8/TIMP-1 molar ratio was significantly elevated in men with subclinical atherosclerosis compared to those without (Tuomainen et al. 2007). Moreover, the serum MMP-8/TIMP-1 ratio was elevated in ACS patients compared to controls, and the ratio was higher in non-STEMI patients compared to STEMI patients (Pussinen et al. 2013). Ultimately, the serum MMP-8/TIMP-1 ratio positively associated with ischemic stroke severity estimated using NIHSS (Palm et al. 2018).

An elevated active and total MMP-8 were measured in the ruptured MI tissue in heart samples taken from patients who died from infarct rupture when compared to control myocardial tissue obtained from MI patients (van den Borne et al. 2009). Therefore, the major source of systemic MMP-8 after an acute event may be derived from infarcted myocardium, not from a plaque rupture.

**Prognostic value of serum MMP-8 in CVD**

An elevated serum MMP-8 level associated with AMI, CVD, death from CHD, or any-cause death during a 10-year follow-up (Tuomainen et al. 2007). Serum MMP-8 and the MMP-8/TIMP-1 ratio associated with an increased risk for an
Review of the literature

incident CVD event during a 10-year follow-up (Tuomainen et al. 2014). Additionally, elevated levels of MMP-8 in the plaque associated with a systemic CVD event during a 3-year follow-up among patients with carotid atherosclerotic disease (Peeters et al. 2011). Furthermore, a high serum MMP-8 level associated with mortality and increased the risk for CVD events during a 13-year follow-up, although serum MMP-8 did not associate with incident stroke (Kormi et al. 2017).

**MMP-8 in periodontitis**

MMP-8 represents the main collagenase in periodontally diseased gingival tissue (Sorsa et al. 2016) and increased levels of saliva MMP-8 appear strongly associated with periodontitis (Sorsa et al. 2016, Miller et al. 2006, Gursoy et al. 2010, Gursoy et al. 2013, Zhang et al. 2018) and its severity (Rathnayake et al. 2013). In addition to saliva, MMP-8 is a major MMP in GCF, periodontal tissue, PISF, and mouth rinse (Sorsa et al. 2004, Sorsa et al. 2006, Sorsa 2011, Sorsa et al. 2016, Marcaccini et al. 2010). Successful periodontal treatment preventing the progression of periodontitis reduced oral MMP-8 concentrations (Chen et al. 2000, Marcaccini et al. 2010). MMP-8 measured from GCF or saliva correlated with gingival bleeding (Chen et al. 2000, Salminen et al. 2014) and ABL as well as PPD ≥ 4 mm (Gursoy et al. 2013). Qualitative and quantitative point-of-care (POC) tests have already been developed (Sorsa et al. 2016) and enjoyed clinical use.

An elevated saliva MMP-8/TIMP-1 ratio has been observed in patients with periodontitis (Rathnayake et al. 2013, Gursoy et al. 2010). The expression of the MMP-8/TIMP-1 ratio was even higher in periodontitis patients with type-2 diabetes compared to nondiabetic periodontitis patients (Bastos et al. 2017).

Moreover, systemically elevated plasma MMP-8 concentrations have also been observed in periodontitis patients compared to healthy controls, whereby periodontal treatment decreased these levels significantly (Marcaccini et al. 2009).

**The effect of smoking on oral and systemic MMP-8**

Earlier studies on the effect of smoking on MMP-8 in oral fluids provide a snapshot of the various complexities associated with smoking. Studies exist whereby MMP-8 measured from oral fluids are lower (Heikkinen et al. 2010, Mäntylä et al. 2006, Liede et al. 1999, Rathnayake et al. 2013), higher (Gupta et al. 2016), or similar (Raitio et al. 2005, Nishida et al. 2008, Liu et al. 2016, Rathnayake et al. 2015) in smokers compared to nonsmokers. In smokers’
saliva MMP-8 did not differ in subjects with periodontitis compared to those without, whereas the MMP-8/TIMP-1 ratio identified periodontitis patients even among smokers (Gursoy et al. 2010).

Elevated levels of serum MMP-8 and the MMP-8/TIMP-1 ratio have been observed in smokers (Lauhio et al. 2016, Aquilante et al. 2007). However, in other studies no significant differences in the plasma or serum MMP-8 concentrations were observed based on smoking status (Djuric et al. 2010, Raitio et al. 2005).

**Other factors affecting MMP-8**

In oral fluids taken from type-2 diabetic patients, elevated levels of MMP-8 were repeatedly measured according to a recent systematic review, although discrepancies do exist (de Morais et al. 2018). The MMP-8 expression in gingival biopsies was higher in type-2 diabetic patients compared to nondiabetic periodontitis patients (Bastos et al. 2017).

In contrast, plasma MMP-8 did not differ according to age in subjects without significant CVD (Bonnema et al. 2007). A three-month atorvastatin therapy significantly reduced serum MMP-8 and the MMP-8/TIMP-1 ration in hypercholesterolemic and type-2 diabetic patients (Kadoglou et al. 2014), possibly mediated due to the anti-inflammatory effects of statins, such as the inhibition of MMPs (Luan et al. 2013). Additionally, serum MMP-8 and the MMP-8/TIMP-1 ratio were elevated in obese compared to normal weight individuals (Lauhio et al. 2016).

### 2.6.2 MMP-9 AS A BIOMARKER FOR CVD AND PERIODONTITIS

**MMP-9 in atherosclerosis and CVD**

Elevated levels of MMP-9 have been found in unstable atherosclerotic plaques alongside an increased amount of collagen and a higher number of SMCs (Brown et al. 1997, Peeters et al. 2011). An increased amount of MMP-9 was observed in expansively remodeled atherosclerotic plaques compared to constrictively remodeled cross-sections (Pasterkamp et al. 2000). This phenomenon was detected through immunostaining from the cap and shoulder regions and the medial border of the plaque (Pasterkamp et al. 2000). In these stainings the presence of MMP-9 strongly associated with the presence of macrophages, whereby MMP-9 was more prevalent in the plaque and not in the adventitia (Pasterkamp et al. 2000).
In addition, serum and plasma MMP-9 concentrations were elevated in CAD (Noji et al. 2001), with the highest levels found in a 3-vessel CAD compared to a 1- or 2-vessel CAD. Thus, serum MMP-9 increases with the severity of the coronary stenosis (Kalela et al. 2002), despite the lack of statistically significant differences between 1- and 2-vessel CAD (Kalela et al. 2002). Additionally, an elevated amount of active MMP-9 and a high expression of MMP-9 occurs in enlarged aneurysms (Pasterkamp et al. 2000).

The plasma MMP-9 concentrations were significantly elevated in UAP and AMI patients compared to controls or stable-effort angina pectoris patients (Kai et al. 1998, Fukuda et al. 2006, Brown et al. 1995, Inokubo et al. 2001, Buduneli et al. 2011), and an elevated serum MMP-9 significantly associated with plaque rupture (Fukuda et al. 2006). In addition to elevated serum MMP-9 levels, the serum MMP-9/TIMP-1 ratio was significantly elevated in STEMI patients compared to controls (Tan et al. 2008). Moreover, elevated MMP-9 levels in oral fluids were also reported in AMI patients (Floriano et al. 2009).

Plasma MMP-9 levels were significantly elevated in stroke patients compared to asymptomatic patients (Turu et al. 2006). An association between elevated levels of plasma MMP-9 and hemorrhagic transformation (HT) of cerebral infarction was found (even after adjusting for potential confounders and the final infarct volume), indicating the role played by the MMP-9 pathophysiology of HT following ischemic stroke (Castellanos et al. 2003).

Systemic MMP-9 levels remained elevated for one week after MI in a rabbit model (Romanic et al. 2001). Persistent levels after an acute event suggest another source of MMP-9 beyond the ruptured plaque. Active and total MMP-9 were higher in patients who died due to myocardial rupture compared to the control myocardial tissue from MI patients (van den Borne et al. 2009). Moreover, the MMP-9 expression increased from the peripheral blood mononuclear cells (PBMCs) in AMI patients compared to healthy controls (Fang et al. 2010). Consequently, ischemic myocardium or PBMCs also represent propable sources of systemically elevated MMP-9.

**Prognostic value of systemic MMP-9 in CVD**

Plasma MMP-9 concentrations associated with an increased risk for MI as well as all-cause and CVD mortality in CAD patients during follow-up (Blankenberg et al. 2003, Cavusoglu et al. 2006, Hansson et al. 2011). However, in chronic heart failure (CHF) patients plasma MMP-9 did not predict all-cause mortality in a 2.5-year follow-up (Frantz et al. 2008).
**MMP-9 in periodontitis**
Saliva MMP-9 was higher in periodontitis patients than among controls (Gursoy et al. 2013, Marcaccini et al. 2010). Additionally, saliva MMP-9 provided a high predictive value in periodontitis in targeted proteomics (Bostanci et al. 2018).

Increased systemic MMP-9 concentrations have been observed in periodontitis patients (Marcaccini et al. 2009). Moreover, plasma MMP-9 concentrations and the MMP-9/TIMP-1 ratio were significantly higher in chronic periodontitis patients with atherosclerosis compared to controls, confirmed using the carotid intima-media thickness (Söder et al. 2009). Periodontal treatment reduces systemic inflammatory markers, and the plasma MMP-9 levels significantly decreased following comprehensive periodontal treatment (Behle et al. 2009, Marcaccini et al. 2009).

**The effect of smoking on oral and systemic MMP-9**
The total oral saliva MMP-9 levels appeared lower in smokers compared to nonsmokers, whereas active-form MMP-9 levels did not differ (Raitio et al. 2005). In other studies saliva or GCF MMP-9 did not differ significantly according to smoking status (Nishida et al. 2008, Liu et al. 2016, Rathnayake et al. 2015).

In plasma elevated levels of MMP-9 were measured among smokers compared to nonsmokers (Turu et al. 2006, Sundström et al. 2004a, Nakamura et al. 1998, Ozcaka et al. 2011). The serum pro-MMP-9 levels were significantly higher in smokers compared to nonsmokers, whereas differences in active-form MMP-9 were not found in the serum (Raitio et al. 2005). The plasma MMP-9 concentrations were related to the duration of smoking history. That is, those who smoked for over 20 years exhibited significantly higher levels of MMP-9 compared to those who smoked for less than 20 years (Nakamura et al. 1998).

**Other factors affecting MMP-9**
Elevated systemic levels of MMP-9 have been observed in diabetic patients (Sundström et al. 2004a, Ebihara et al. 1998, Derosa et al. 2007, Signorelli et al. 2005), whereas disparate results also exist (Papazafiropoulou et al. 2010, Lee et al. 2005). Plasma MMP-9 was elevated in diabetes patients with peripheral arterial disease (PAD) compared to those without (Signorelli et al. 2005). This finding suggests that the dysregulation of MMPs is important in the development of diabetic vascular complications. Serum MMP-9
concentrations decreased significantly during treatment (for 26 weeks) with the antidiabetic drug rosiglitazone for type-2 diabetes mellitus (T2DM) patients (Haffner et al. 2002).

The plasma MMP-9 and MMP-9/TIMP-1 ratio were lower among older than among younger subjects without significant CVD (Bonnema et al. 2007). Patient sex did not affect the circulating MMP-9 concetrations in healthy volunteers (Taybjee et al. 2005). Yet, both elevated and diminished systemic MMP-9 levels were measured in hypertensive subjects (Niemierska et al. 2016, Taybjee et al. 2004, Taybjee et al. 2004, Li-Saw-Hee et al. 2000), as well as among patients receiving antihypertensive medication (Taybjee et al. 2004, Li-Saw-Hee et al. 2000, Sundström et al. 2004a).

Simvastatin significantly decreased the plasma MMP-9 concentrations and activity in CAD patients compared to patients receiving a placebo and dietary counselling alone (Koh et al. 2002). A similar decreasing trend in the serum MMP-9 due to pravastatin therapy was observed among healthy men (Kalela et al. 2001). Additionally, statins have been shown to decrease MMP-9 secretion in cell experiments (Bellosta et al. 1998, Luan et al. 2003).

Furthermore, the circulating MMP-9 levels differed according to ethnicity (Afro-Caribbean, Asian, Caucasian, Far Eastern); the lowest concentrations were observed in Far Eastern subjects (Taybjee et al. 2005). No intrapersonal diurnal variation was found in the systemic MMP-9 levels when healthy individuals were sampled at four time points (at 8:00, 12:00, 18:00, and 22:00) (Taybjee et al. 2005).

The association between MMP-9 expression and the intravascular presence of *C. pneumoniae* antigen was found (Arno et al. 2005). In addition, the MMP-9 activity increased due to *C. pneumoniae* in atherosclerotic plaques (Choi et al. 2002). Genetic variation in the promoter of the MMP-9 gene influences its expression and protein levels (Medley 2004). This C-1562T polymorphism associated both with the plasma MMP-9 concentrations in CAD patients (Blankenberg et al. 2003) and the degree of stenosis (Zhang 1999).

### 2.6.3 TIMP-1 AS A BIOMARKER FOR CVD AND PERIODONTITIS

**TIMP-1 in atherosclerosis and CVD**

TIMP-1 expression appears elevated in the calcification areas of atherosclerotic plaques (Orbe et al. 2003). TIMP-1 is potentially proatherogenic, as demonstrated in apolipoprotein E (ApoE) deficient mice.
susceptible to atherosclerosis kept on a cholesterol-rich diet (Silence et al. 2002). The overexpression of TIMP-1 produced a preventive effect towards aortic aneurysm formation and rupture (Allaire et al. 1998). In addition, TIMP-1 stimulates SMC proliferation in a dose-dependent manner (Akahane et al. 2004), while TIMP-1 appears to promote inflammation (Apparailly et al. 2001).

Systemic TIMP-1 concentrations were significantly elevated in CAD (Noji et al. 2001, Pradhan-Palikhe et al. 2010) and ACS (Pussinen et al. 2013, Inokubo et al. 2001, Cavusoglu et al. 2006). STEMI patients exhibited significantly elevated serum TIMP-1 levels compared to controls (Tan et al. 2008), whereas such concentrations were lower in non-STEMI patients (Pussinen et al. 2013). Furthermore, increased levels of serum TIMP-1 have been associated with PAD (Beaudeux et al. 2003) and ischemic stroke (Palm et al. 2018, Lorenzl et al. 2003). Yet, saliva TIMP-1 levels did not differ significantly between AMI and non-AMI patients (Rathnayake et al. 2015).

TIMP-1 regulates the left ventricular (LV) structure and function, and may serve as a useful marker for evaluating the healing process (Hirohata et al. 1997). Serum TIMP-1 reached its highest value on day 14 following AMI (Hirohata et al. 1997), with concentrations decreasing significantly during recovery (Pussinen et al. 2013).

**Prognostic value of systemic TIMP-1 in CVD**

Serum TIMP-1 represents an independent predictor of CVD events, including CVD death and stroke (West et al. 2008, Hansson et al. 2011, Kormi et al. 2017). Elevated acute (Lubos et al. 2006, Kormi et al. 2017, Pussinen et al. 2013, Frantz et al. 2008, Cavusoglu et al. 2006) and recovery-phase (Pussinen et al. 2013) serum and plasma TIMP-1 concentrations have been associated with a future CVD event, all-cause mortality, and cardiac death. In the large-scale FINRISK study, serum TIMP-1 was the strongest biomarker in CVD, while adding TIMP-1 concentrations to the Framingham risk profile improved the predictive value of the model assessing AMI and death (Kormi et al. 2017).

**TIMP-1 and periodontitis**

Significantly lower saliva TIMP-1 levels have been observed in periodontitis patients compared to controls (Nizam et al. 2014, Gursoy et al. 2010). However, in another study TIMP-1 concentrations measured from GCF did not differ between chronic periodontitis patients and healthy controls, and no change was observed during a three-month follow-up period (Marcaccini et al. 2010).
The plasma TIMP-1 levels were significantly higher in patients with chronic periodontitis and an elevated carotid intima-media thickness compared to controls (Söder et al. 2009). However, in periodontitis patients free of atherosclerosis or other systemic diseases the plasma TIMP-1 concentrations did not differ when compared to healthy controls (Marcaccini et al. 2009). Furthermore, periodontal treatment did not affect the plasma TIMP-1 levels (Marcaccini et al. 2009).

**The effect of smoking on oral and systemic TIMP-1**
Saliva TIMP-1 was elevated in smokers compared to nonsmokers both in periodontitis patients and control subjects (Gursoy et al. 2010); by contrast, serum TIMP-1 was significantly lower in chronic periodontitis patients who smoke (Ozcaka et al. 2011). In studies without information about periodontal status smokers exhibited elevated plasma TIMP-1 levels (Sundström et al. 2004b), and the serum TIMP-1 inversely correlated with a history of smoking (Lubos et al. 2006).

**Other factors affecting TIMP-1**
The plasma TIMP-1 concentrations were significantly elevated in type-2 diabetic subjects compared to controls (Derosa et al. 2007, Lee et al. 2005), whereas another study found such levels were significantly lower (Papazafiropoulou et al. 2010).

TIMP-1 expression measured from gingival biopsies were lower in type-2 diabetic periodontitis patients than in nondiabetic periodontitis patients (Bastos et al. 2017).

Male gender, age, and BMI all associated with increased plasma TIMP-1 concentrations (Sundström et al. 2004b). The MMPs and TIMPs profiles may change with age, thus favoring ECM accumulation among the elderly (Niemirksa et al. 2016). Plasma TIMP-1 increased significantly with age among CVD-free subjects (Bonnema et al. 2007). In another study, patient sex did not affect circulating TIMP-1 levels among healthy volunteers (Taybjee et al. 2005).

Systemically elevated (Taybjee et al. 2004a, Taybjee et al. 2004b, Laviades et al. 1998) and decreased (Li-Saw-Hee et al. 2000) TIMP-1 concentrations have been measured in hypertensive adults. In addition, plasma TIMP-1 increased with the total/high-density lipoprotein (HDL) cholesterol ratio (Sundström et al. 2004b). Moreover, alcohol intake was inversely associated with the plasma TIMP-1 concentrations (Sundström et al. 2004b), and circulating TIMP-1
increased during maximal exhaustion treadmill tests in healthy volunteers (Taybjee et al. 2005). Intrapersonal diurnal variation in systemic TIMP-1 was not observed when healthy individuals were sampled at four time points (8:00, 12:00, 18:00, and 22:00) (Taybjee et al. 2005).

Patients receiving statin therapy had lower serum and plasma TIMP-1 levels compared to those no receiving statins (Lubos et al. 2006, Koh et al. 2002). However, patients undergoing pravastatin treatment had a higher TIMP-1 immunoreactivity in their carotic plaques compared to those without statins (Crisby et al. 2001). No significant differences were observed according to other medications (diuretics, beta-blockers, angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, and anti-inflammatories) and the MMP or TIMP plasma profiles (Bonnema et al. 2007). However, in another study losartan increased the secretion of TIMP-1 from human VSMCs (Papakonstantinou et al. 2001).

2.6.4 MPO AS A BIOMARKER FOR CVD AND PERIODONTITIS

**MPO in atherosclerosis and CVD**

An elevated systemic MPO concentration has repeatedly associated with CAD and its severity (Zhang et al. 2001, Ruef et al. 2006, Rebeiz et al. 2011, Teng et al. 2017, Peacock et al. 2011). Moreover, an elevated saliva MPO has been associated with AMI (Floriano et al. 2009). Serum MPO associated both with ischemic stroke and stroke severity measured by NIHSS (Palm et al. 2018). The serum MPO levels of ischemic stroke patients compared to controls were significantly higher on the first day following the event, although on the fifth day following an acute event the serum MPO decreased approaching levels measured for controls (Tay et al. 2015).

**Prognostic value of serum MPO**

An elevated plasma MPO associated with an increased risk for CVD mortality, particularly when combined with the CRP concentrations (Heslop et al. 2010). Furthermore, an increased serum MPO associated with an elevated risk for stroke and mortality in a 13-year follow-up (Kormi et al. 2017). Serum MPO associated with death during a follow-up among ischemic stroke patients (Tay et al. 2015).

**MPO in periodontitis**

Higher concentrations of MPO in GCF and saliva have been observed in periodontitis patients (Nizam et al. 2014, Marcaccini et al. 2010), whereby such levels and the MPO activity significantly decreased following periodontal
therapy during a three-month follow-up period (Marcaccini et al. 2010, Meschiari et al. 2013). Moreover, saliva MPO correlated with BOP, PPD, and clinical periodontal diagnosis (Rathnayake et al. 2015).
Elevated systemic MPO concentrations have been measured in periodontitis patients compared to controls (Nizam et al. 2014), and the plasma MPO concentrations significantly decreased following periodontal therapy (Behle et al. 2009).

**The effect of smoking on MPO**

Smokers exhibited an elevated systemic MPO compared to nonsmokers (Andelid et al. 2007, van Eeden et al. 2000, Lavi et al. 2007). On the one hand, systemic MPO levels increased during a six-year follow-up period among smokers, but decreased as the time since cessation increased, most likely reflecting the systemic inflammatory state caused by smoking (Andelid et al. 2007). However, interestingly, smokers whose status was confirmed through cotinine measurements exhibited significantly lower serum MPO levels compared to nonsmokers (Ebersole et al. 2014). Yet, in another study smokers exhibited a higher serum MPO compared to nonsmokers, when both groups had chronic periodontitis (Ozcaka et al. 2011). No significant differences in oral MPO were measured between smokers and nonsmokers (Rathnayake et al. 2015). Yet, smoking led to the loss of MPO granules in human neutrophils, thereby impairing neutrophil functioning, possibly serving as an explanatory factor for smokers’ chronic inflammation and an inability to react to a microbial challenge (Zappacosta et al. 2011).

**Other factors affecting MPO**

Atorvastatin reduced the plasma MPO levels significantly together with CRP following a 12-week treatment period (Mayyas et al. 2018). In addition, statins decreased the MPO levels measured from GCF and improved periodontal measurements, also describing the anti-inflammatory effects of statins locally in the periodontium (Cicek et al. 2016).

**2.6.5 SUMMARY OF ORAL AND SYSTEMIC BIOMARKERS FOR PERIODONTITIS AND CVD**

**CRS in estimating periodontitis**

The cumulative risk score (CRS) combines the measurements of saliva MMP-8, interleukin 1β (IL-1β), and *P. gingivalis*, representing as a novel diagnostic index of periodontitis in epidemiological studies without a clinical oral and periodontal examination (Gursoy et al. 2011). IL-1β is a potent pro-inflammatory mediator primarily produced by monocytes, macrophages, and dendritic cells during periodontal inflammation (Gursoy et al. 2009). CRS is
beneficial when a trained dental professional is unavailable, but saliva samples can be collected in large studies. CRS describes the periodontal pathogen burden, inflammation, and tissue degradation (Gursoy et al. 2011). Furthermore, CRS associates with moderate and severe periodontitis more than any of these markers on their own (Gursoy et al. 2011, Salminen et al. 2014).

**Other biomarkers for CVD**

In addition to MMPs, TIMPs, and MPO, several biomarkers related to CVD have been investigated. Biomarkers describing the myocardial injury include cardiac troponin, high-sensitivity cardiac troponin (Hs-cTn), and the heart-type fatty acid binding protein (H-FABP) (Wang et al. 2017). Biomarkers of inflammation include high-sensitivity C-reactive protein (hsCRP) and fibrinogen (Wang et al. 2017). Biomarkers related to platelet activation consist of lipoprotein-associated phospholipase A₂ (Lp-PLA₂) and soluble CD40 ligand (sCD40L) (Wang et al. 2017).

Furthermore, saliva as a sample material has been studied abundantly in CVD diagnostics. Biomarkers such as CRP, MPO, MMP-9, and IL-1β measured from saliva were investigated in the diagnosis of AMI (Christodoulides et al. 2012). However, the use of saliva in CVD diagnostics remains challenging, because saliva contains many oral-derived enzymes and inhibitors (Miller et al. 2010).

**Summary of biomarkers for periodontitis and CVD**

Table 1 summarizes current knowledge on oral and systemic biomarkers (MMP-8, MMP-9, TIMP-1, and MPO) in ACS, ischemic stroke, periodontitis, and in smokers. Quite a few studies exist on these biomarkers in ischemic stroke, while studies considering oral biomarkers in ischemic stroke remain scarce. In addition, quite a few studies exist on oral biomarkers for ACS. Additionally, studies about smoking appear inconsistent.
Table 1. Summary of current literature about oral and systemic MMP-8, MMP-9, TIMP-1, and MPO in ACS, ischemic stroke, periodontitis, and smokers.

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<tbody>
<tr>
<td></td>
<td>Oral</td>
<td>↑Floriane et al. 2009 —Rathnayake et al. 2015</td>
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<tr>
<td></td>
<td>Oral</td>
<td>—Rathnayake et al. 2015</td>
<td></td>
<td></td>
<td>↑Sundström et al. 2004b —Ozaka et al. 2011</td>
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<tr>
<td></td>
<td>Oral</td>
<td>↑Floriane et al. 2009</td>
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</table>

Images modified from Darveau 2010 and Libby et al. 2010. MMP-8 = matrix metalloproteinase-8, MMP-9 = matrix metalloproteinase-9, TIMP-1 = tissue inhibitor of matrix metalloproteinase-1, MPO = myeloperoxidase. ↑ = elevated levels, ↓ = decreased levels, ─ = no significant differences.
3 AIMS OF THE STUDY

Broadly, this study aimed to explore local and systemic biomarkers related to the degradation of the extracellular matrix (ECM) in periodontitis and cardiovascular diseases (CVDs). Furthermore, the effect of smoking on biomarker diagnostics was examined. Finally, the effect of the cardiac status on the salivary diagnostics of periodontitis and the effect of periodontitis on serum biomarker diagnostics in ACS were investigated, ultimately aiming to increase knowledge regarding the association between periodontitis and CVD.

The specific aims for this study were to investigate:

I. The diagnostic value of serum MMP-8, MMP-9, TIMP-1, and MPO in ACS and ischemic stroke.

II. The prognostic value of serum MMP-9 and the MMP-9/TIMP-1 ratio and their Δ values for major adverse cardiovascular events (MACE), that is, a new cardiac event or CVD death.

III. The diagnostic value of saliva MMP-8, MMP-9, TIMP-1, and MPO in periodontitis.

IV. The effect of smoking, periodontitis, and CVD on biomarker diagnostics.
## 4 STUDY SUBJECTS AND METHODS

### 4.1 STUDY SUBJECTS AND DESIGNS

Table 2. Summary of study subjects and designs.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>Sample material</th>
<th>Collection time</th>
</tr>
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<tbody>
<tr>
<td><strong>Triangel study (I)</strong></td>
<td>Cross-sectional, case-control, longitudinal study</td>
<td>Serum without clot activators</td>
<td>From patients within 24 hours after an acute ACS event</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>From controls collected by a research nurse during a home visit</td>
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<td></td>
<td>157 (45.8%) patients were resampled following a 6-month recovery period</td>
</tr>
<tr>
<td><strong>Ludwigshafen study (II)</strong></td>
<td>Case-control study</td>
<td>Serum sample without clot activators</td>
<td>From patients within 24 hours following a hospital admission</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>From controls during a laboratory visit</td>
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<tr>
<td></td>
<td></td>
<td>Paraffin-stimulated whole saliva</td>
<td>From patients within 24 hours following a hospital admission</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>From controls during a laboratory visit</td>
</tr>
<tr>
<td><strong>Parogene study (III &amp; IV)</strong></td>
<td>Cross-sectional, cohort study</td>
<td>Blood sample, serum</td>
<td>During coronary angiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paraffin-stimulated whole saliva</td>
<td>Before oral examination, 37 to 224 days following angiography</td>
</tr>
</tbody>
</table>
Table 3. Main characteristics of subjects in the Triangel, Ludwigshafen, and Parogene studies.

<table>
<thead>
<tr>
<th>Case</th>
<th>Control</th>
<th>Case</th>
<th>Control</th>
<th>Case</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD), p value</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (in years)</strong></td>
<td>63.3 (9.2)</td>
<td>63.0 (9.2), NS</td>
<td>68.2 (9.7)</td>
<td>69.1 (5.2), NS</td>
<td>63.0 (9.5)</td>
</tr>
<tr>
<td><strong>Sex (male)</strong></td>
<td>275 (78.7)</td>
<td>254 (77.9), NS</td>
<td>53 (54.1)</td>
<td>53 (53.0), NS</td>
<td>118 (72.4)</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td>46 (13.4)</td>
<td>–</td>
<td>31 (31.6)</td>
<td>14 (14.0), 0.003</td>
<td>36 (22.1)</td>
</tr>
<tr>
<td><strong>Dyslipidemia</strong></td>
<td>77 (22.5)</td>
<td>–</td>
<td>75 (76.5)</td>
<td>67 (67.0), NS</td>
<td>120 (73.6)</td>
</tr>
<tr>
<td><strong>Smoking (current)</strong></td>
<td>55 (16.0)</td>
<td>66 (21.0), NS</td>
<td>28 (28.6)</td>
<td>12 (12.0), 0.004</td>
<td>17 (10.4)</td>
</tr>
</tbody>
</table>

Case refers to ACS patients in the Triangel and Parogene studies, and ischemic stroke patients in the Ludwigshafen study.

*Refers to hyperlipidemia in the Ludwigshafen study and lipid-lowering medication in the Triangel study.

Statistical significance determined using the independent samples t-test for continuous variables and chi-square test for categorical variables.

SD= standard deviation, NS= not significant.
Study subjects and methods

4.1.1 THE TRIANGEL STUDY (STUDY I)

The Triangel study population consisted of ACS patients (n = 343) admitted to the heart intensive care unit at Lund University Hospital and age- and sex-matched healthy controls (n = 326). Data were collected between March 1999 and April 2002.

The inclusion criteria for both controls and patients were as follows (Pesonen et al. 2009):
- inhabitant of the Lund area.
- age <80 years old.
- no signs of a cognitive intellectual disability.

In addition, controls (n = 326) needed to satisfy the following additional inclusion criteria:
- no previous CVD history (CHD, stroke, or angina pectoris).
- not taking any medications for dyslipidemia, hypertension, or diabetes.

The inclusion criteria for cases (n = 343) were based on an ACS diagnosis. In addition, AMI diagnosis (n = 235) was determined if two of the following criteria were fulfilled:
- chest pain related to exercise and lasting more than 20 minutes.
- changes in an electrocardiography (ECG), such as ST elevations followed by T-wave inversion or new Q waves.
- an increase in creatine kinase-MB (CK-MB) to more than twice the upper limit of the normal value >5 μg/l.

UAP (n = 108) was diagnosed if the patient presented with two of the following criteria:
- continuous chest pain.
- ST-segment depression in ECG (<1 mm).
- CK-MB elevation (5 < CK-MB < 10 μg/l).
- Troponin T (TnT) elevation (0.05 < TnT < 0.10 μg/l).

Follow-up

During a 6-month recovery period (median, 350 days; interquartile range (IQR), 434 days), 157 (45.8%) patients were resampled. All subjects were followed-up for an average of 6 years (range, 4.56–7.13 years) until experiencing a major adverse cardiac event (MACE) or until the end of the study period. MACE included CVD death (fatal MACE, n = 61) and a new ACS event (nonfatal MACE, n = 89). Recovery samples from patients suffering
MACE were obtained from 14 patients with a fatal MACE and from 49 patients with a nonfatal MACE. Information regarding smoking habits (nonsmoker vs. current smoker) was collected via questionnaires by trained nurses. In the ACS group, 55 patients (16%) and 66 controls (21%) were current smokers. We did not have information about the smoking status for 52 ACS patients and for 12 controls.

4.1.2 THE LUDWIGSHAFEN STUDY (STUDY II)

The Ludwigshafen study is a subsample of the larger GENESIS study, an ongoing study on the determinants of ischemic stroke collected in the city of Ludwigshafen, Germany. GENESIS consists of subjects recruited to the Ludwigshafen Stroke Study (LuSST), a population-based stroke registry study initiated in 2006 (Palm et al. 2010). This specific Ludwigshafen study population was recruited between July 2010 and October 2011. Trained nurses interviewed study subjects, including questions about their smoking status, the number of remaining teeth, and their infectious disease history using questionnaires. In addition, the following question was posed: “Has a dentist diagnosed you as having periodontitis?” Two stroke patients were retrospectively diagnosed with a recurrent stroke, and were thus excluded from the study.

The inclusion criteria for controls (n = 100) consisted of the following:
- inhabitant of the Ludwigshafen area.
- no previous history of CVD (stroke or AMI).
- age between 18 and 80 years.

The inclusion criteria for ischemic stroke patients (n = 98) consisted of the following:
- incident stroke or transient ischemic attack.
- no clinical or laboratory signs of acute infection at the time of the stroke.

Stroke diagnosis relied on World Health Organization definitions (Hatano 1976), or rapidly developing clinical stroke symptoms and/or a disturbance in the cerebral function lasting >24 hours or leading to death without another origin or explanations as described in further detail elsewhere (Palm et al. 2010). Ischemic stroke patients were distinguished from hemorrhagic stroke using brain imaging.

Smoking status

Smoking status was categorized as follows:
- current smokers (n = 40 consisting of 12 controls and 28 cases).
- nonsmokers (n = 158), further categorized as follows:
Study subjects and methods

- former smokers (n = 75, consisting of 45 controls and 30 cases).
- never smokers (n = 83, consisting of 43 controls and 40 cases).

The calculation of the cumulative risk score (CRS)
The following measures were used in the Ludwigshafen study to calculate the cumulative risk score (CRS): saliva MMP-8 (tertiles), saliva IL-1β (tertiles), and saliva P. gingivalis (three groups: zero level, below median levels, and above median levels). The subscores were multiplied by one another to calculate the CRS scores as I referring to a low risk (cumulative subscores of 1, 2, and 3), II as a medium risk (cumulative subscores of 4, 8, and 9), and III as a high risk (cumulative subscores of 12, 18, and 27) of having periodontitis.

4.1.3 THE PAROGENE STUDY (STUDY III & IV)
The Parogene cohort (n = 508) represents a subsample of the larger Corogene cohort (n = 5809) recruited at the Helsinki University Central Hospital between 2006 and 2008 (Vaara et al. 2012). All subjects in the Parogene study presented with an indication of coronary angiography and underwent a detailed oral examination. The oral examination included periodontal probing pocket depth (PPD) measurements from six sites for each tooth and BOP measurements. ABL was calculated from digital panoramic radiographs. Both serum and saliva samples were available for 481 patients. The cohort was described in more detail previously (Buhlin et al. 2011).

The inclusion criteria consisted of the following:
- indication of coronary angiography.
- Finnish origin.
- no previous heart transplantation or blood transfusions.

Periodontal diagnosis
Periodontitis (n = 285) was determined if both of the following criteria were fulfilled:
- ABL (mild to severe, specifically whereby ABL was present in the cervical third of the root to total ABL).
- PPD reached ≥4 mm along ≥4 sites.

Patients without periodontitis (n = 196) consisted of the following:
- periodontally healthy.
- gingivitis patients.
- edentulous patients.
**CAD diagnosis** fell with the following categories. A non-CAD diagnosis (n = 115) referred to patients with <50% stenosis of all coronary arteries in angiography. A stable CAD diagnosis (n = 175) consisted of patients with >50% stenosis in angiography.

An ACS diagnosis (n = 163) fell into three categories, as follows:
- ≥50% stenosis in at least one coronary artery.
- episode of chest pain typical for ischemia.
- elevated cardiac enzymes.

An ACS-like diagnosis with no significant CAD (n = 28) consisted of Takotsubo patients.

**Follow-up**
The follow-up period was calculated from the angiography date through June 2014 or alternatively to the date of death. The median (IQR) follow-up time was 2469 (2402–2520) days. During this 6.8-year follow-up period, 54 patients died due to any reason, and 34 of them died due to cardiac-related causes.

**Smoking status**
The smoking status was categorized as follows:
- never smoker (n = 227).
- quit smoking more than one year ago (n = 150).
- quit less than one year ago (n = 47).
- current smokers (n = 56).

Pack years of smoking (based on information available for 419 (82%) patients) was categorized as follows:
- 0 pack years (n = 227).
- 1 to 20 pack years (n = 94).
- >20 pack years (n = 98).

<table>
<thead>
<tr>
<th>Pack years of smoking</th>
<th>0</th>
<th>1–20</th>
<th>&gt;20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time since cessation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>227</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Quit &gt;1 year ago</td>
<td>0</td>
<td>57</td>
<td>51</td>
</tr>
<tr>
<td>Quit &lt;1 year ago</td>
<td>0</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Current</td>
<td>0</td>
<td>19</td>
<td>28</td>
</tr>
</tbody>
</table>
## 4.2 METHODS

Table 4. Summary of the methods used in this thesis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Clinical methods</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical oral examination</td>
<td>PPD from 6 sites of each tooth, BOP from 4 sites of each tooth</td>
<td>III, IV</td>
</tr>
<tr>
<td>Oral radiographic examination</td>
<td>ABL calculation [no or mild (ABL in the cervical third of the root), moderate (ABL in the middle third of the root), severe (ABL in the apical third of the root), and total]</td>
<td>III, IV</td>
</tr>
<tr>
<td>Angiography</td>
<td></td>
<td>III, IV</td>
</tr>
<tr>
<td>ECG</td>
<td></td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Brain imaging</td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>Medical record data on cardiometabolic medications</td>
<td>Medications used before hospitalization and prescribed during hospitalization</td>
<td>III</td>
</tr>
<tr>
<td><strong>Laboratory methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>MMP-9</td>
<td>I, III, IV</td>
</tr>
<tr>
<td></td>
<td>TIMP-1</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td>MPO</td>
<td>II, III, IV</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Antibody levels to <em>A. actinomycetemcomitans</em> and <em>P. gingivalis</em></td>
<td>II</td>
</tr>
<tr>
<td>Time-resolved immunofluorometric assay (IFMA)</td>
<td>MMP-8</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Zymography</td>
<td>MMP-9</td>
<td>I</td>
</tr>
<tr>
<td>Quantitative polymerase chain reaction (qPCR)</td>
<td><em>A. actinomycetemcomitans</em>, <em>P. gingivalis</em>, <em>T. forsythia</em>, and <em>P. intermedia</em></td>
<td>II</td>
</tr>
<tr>
<td>Limulus amebocyte lysate assay (LAL)</td>
<td>LPS activity</td>
<td>II</td>
</tr>
<tr>
<td><strong>Statistical analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Independent samples t-test (Student’s t-test)</td>
<td>Normally distributed continuous data, two groups</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Analysis of Variance (ANOVA) test</td>
<td>Normally distributed continuous data, several groups</td>
<td>I</td>
</tr>
<tr>
<td>Chi-square test ($\chi^2$ test) (Nonparametric) Mann-Whitney U test</td>
<td>Categorical data</td>
<td>I, II, III, IV</td>
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<td>Continuous variables with a nonnormal distribution, two groups</td>
<td>I, III</td>
</tr>
<tr>
<td>Kruskal-Wallis test</td>
<td>Continuous variables with a nonnormal distribution, several groups</td>
<td>I, II</td>
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<tr>
<td>Wilcoxon signed-rank test</td>
<td>Continuous variables with a nonnormal distribution, two related samples, paired test</td>
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<td>Receiver operating characteristics (ROC) analysis</td>
<td></td>
<td>I, III, IV</td>
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<td>Cut-off value determinations$^1$</td>
<td>Calculation of sensitivity &amp; specificity pairs</td>
<td>III, IV</td>
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<td>Multivariate logistic regression model</td>
<td>Association analysis</td>
<td>I, II, III, IV</td>
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<tr>
<td>Cox regression model</td>
<td>Survival analysis</td>
<td>I</td>
</tr>
<tr>
<td>Spearman’s correlation analysis</td>
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<td>I, II, III, IV</td>
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<td>Additional data for I, IV</td>
</tr>
<tr>
<td>C statistics</td>
<td></td>
<td>Additional data for I, IV</td>
</tr>
</tbody>
</table>

$^1$The cut-off value determined for the point at which the distance from the left-upper corner of the unit square was the smallest (Habibzadeh et al. 2016).

**Calculation of MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios**
For these calculations, we used the following molecular weights:
- 65 kDa for MMP-8.
- 92 kDa for MMP-9.
- 28 kDa for TIMP-1.
4.2.1 LABORATORY METHODS

Enzyme-linked immunosorbent assay (ELISA)
We used enzyme-linked immunosorbent assay (ELISA) to quantify the concentration of the desired protein.

In our analysis, we used the following commercial ELISA kits for the serum and saliva determinations, using kits according to the manufacturer’s instructions on diluted samples:

- MMP-9–ELISA (GE Healthcare UK Limited, Amersham Place, UK) for the Triangel and Parogene studies.
- TIMP-1–ELISA (R&D Systems, Minneapolis, MN, USA) for the Triangel and Parogene studies.
- TIMP-1 ELISA (Amersham Biotrak, GE Healthcare, Buckinghamshire, UK) for the Ludwigshafen study.
- MPO ELISA (Immundiagnostik, Bensheim, Germany) for the Ludwigshafen and Parogene studies.
- IL-1β ELISA (Amersham Biotrak, GE Healthcare, Buckinghamshire, UK) for the Ludwigshafen study.

Briefly, concentrations were measured by adding samples, standards, and controls in precoated wells (96-well microplate). After washing, the bound protein was measured using a peroxidase-conjugated secondary anti-antibody (targeted towards the desired protein) and developed with hydrogen peroxidase and tetramethylbenzidine. The optical density was measured at a wavelength of 450 nm. The concentration of the measured protein was calculated from the calibration curve, drawn based on standards measured as duplicates.

Multiserotype ELISA against serum IgA and IgG antibodies
In the Ludwigshafen study serum IgA and IgG antibody levels against whole-cell antigens of A. actinomycetemcomitans and P. gingivalis were measured using multiserotype ELISA as described previously (Pussinen et al. 2002). The antigen mixtures contained six strains (representing serotypes a, b, c, d, and e and a nonserotypeable strain) of A. actinomycetemcomitans and three strains (representing serotypes a, b, and c) of P. gingivalis. For A. actinomycetemcomitans–IgG, dilutions of 1/1500 and 1/3000 were used, while for A. actinomycetemcomitans–IgA and P. gingivalis–IgA and –IgG, we used dilutions of 1/100 and 1/200. Following analysis, results were normalized using the reference serum samples applied on each plate.
**Time-resolved immunofluorometric assay (IFMA)**

In all substudies MMP-8 concentrations were determined with a time-resolved immunofluorometric assay (IFMA) (Medix Biochemica, Kauniainen, Finland) according to the manufacturer’s instructions as previously described (Hanemaaijer et al. 1997). The MMP-8-specific monoclonal antibodies were used (8708 and 8706 for the catching and tracer antibody; Medix Biochemica, Kauniainen, Finland). The detection (tracer) antibody was labeled with a fluorescent probe (europium chelate).

**Quantitative real-time polymerase chain reaction (qPCR)**

Quantitative real-time PCR (qPCR) was used to determine the levels of saliva *A. actinomycetemcomitans, P. gingivalis, T. forsythia, and P. intermedia* in the Ludwigshafen study as described previously (Hyvärinen et al. 2009). Results were presented as concentrations (genomes/ml), calculated according to standard curves.

**LPS activity assay**

A *limulus* amebocyte lysate (LAL) assay coupled with a chromogenic substrate (HyCult Biotechnology, Uden, Netherlands) was used to determine the serum and saliva LPS activity in the Ludwigshafen study as previously described (Hyvärinen et al. 2012). The LAL assay utilizes the phenomenon LPS initiating the clotting cascade in the amebocytes of the American horseshoe crab, *Limulus Polyphemus*. Due to the coupled chromogenic substrate, the reaction caused by LPS can be measured using a spectrophotometer. The standard is provided by the manufacturer.

**Gelatin-substrate zymography**

Gelatin-substrate zymography is widely used to detect proteolytically activated gelatinases, particularly MMP-9, based on the molecular weight, thus differentiating the latent and active forms situated in different molecular weights (Kupai et al. 2010). The technique is based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Using this method, MMPs are separated from their endogenous inhibitors, TIMPs, via electrophoresis (Kupai et al. 2010). In the substrate, zymography samples are not boiled nor is any reducing agent used, and the proteins are separated using a standard denaturating SDS-PAGE treatment buffer (Kupai et al. 2010). The SDS inactivates and denaturates the enzymes, and the activation of the latent enzymes in gel is thought to occur due to the dissociation of the Cys residue from Zn (Springman et al. 1990). Following electrophoresis, enzymes are
renaturated with nonionic detergent and subsequently incubated at pH 7.4 at 37°C in the presence of calcium. The MMP activity is detected as digested zones in gel following an incubation period and the bands can be quantified using densitometry.

In the Triangel study, a gelatin zymography was used as previously described (Sorsa et al. 1997). In the zymography analyses, we used the following molecular weights:

- 92 kDa for pro-MMP-9.
- 82 kDa for active MMP-9.
- 72 kDa for pro-MMP-2.
- 64 kDa for active MMP-2.
- >100 kDa for higher gelatinolytic species, such as MMP-9 clustered together with the neutrophil gelatinase-associated lipocalin (NGAL) and MMP-9 linked with TIMP-1.

The gelatin-zymography experiment in the Triangel study was performed using the serum samples as such and using serum samples activated with APMA to detect the *in vitro* activation of MMP-9.

### 4.2.2 STATISTICAL METHODS

The distribution of continuous variables was analyzed before statistical processing. Normally distributed variables were presented as means and standard deviations (SDs), while variables with a skewed distribution were presented as medians and interquartile ranges (IQRs). The analyses used for examining statistical significance are listed in Table 4 above. We set the level of statistical significance to \( p = 0.05 \).

The diagnostic ability—that is, the sensitivity and specificity—of biomarkers were calculated using the receiver operating characteristics (ROC) analysis, from which the area under the curve (AUCs) values were obtained. Sensitivity and specificity pairs were calculated using the cut-off concentrations set to the point of the most minimal distance from the upper-left corner of the ROC analysis unit square (Habibzadeh et al. 2016) (Figure 6). The ROC analysis progressed using C statistics from predicted probabilities with adjusted variables.
Figure 6. Receiver operating characteristics (ROC) curve and determination of the different cut-off values. Modified from Habibzadeh et al. 2016.

Univariate and several multivariate logistic regression models were used to determine the relationships between biomarkers and periodontitis or ACS. The Cox regression model was used to calculate the association of biomarkers with endpoints during follow-up. The Spearman correlation was used for correlation analyses. All analyses were performed using IBM’s SPSS Statistics versions 18 to 24 and SAS version 9.1.3.
5 RESULTS

5.1 SERUM BIOMARKERS ELEVATED IN ACS AND STROKE

The serum biomarkers were significantly elevated in CVD, whereas periodontitis could not be detected using the serum biomarkers (Figure 7). The serum MMP-8 and MMP-9 concentrations, as well as the MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios, were significantly elevated in ACS in both the Parogene and Triangel study populations. Serum TIMP-1 was also significantly elevated, albeit the difference was small in ACS in the Triangel study, but not in the Parogene study population. Furthermore, serum MPO was significantly elevated in ACS and ischemic stroke in the Parogene and Ludwigshafen studies. In stroke patients, serum MMP-8 and the MMP-8/TIMP-1 ratio were also significantly elevated.

Figure 7. The serum MMP-8, MMP-9, TIMP-1, and MPO concentrations (ng/ml) and the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios in periodontitis, acute coronary syndrome (ACS), and ischemic stroke. Values analyzed separately in the Parogene, Triangel, and Ludwigshafen study populations and presented as medians and interquartile ranges (IQRs). Statistical significance determined using the nonparametric Mann-Whitney U test. ** refers to $p < 0.001$ and * refers to $p < 0.05$. 
5.2 **SALIVA BIOMARKER LEVELS DIFFERED ACCORDING TO PERIODONTAL STATUS**

Periodontitis was detected using saliva biomarkers, whereas saliva biomarkers did not differentiate ACS patients from controls (Figure 8). In periodontitis patients, the saliva MMP-8, MMP-9, and MPO concentrations, as well as the MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios, were significantly elevated. Saliva TIMP-1 was significantly lower among periodontitis patients compared to the nonperiodontitis group. In addition, ischemic stroke patients had significantly lower levels of saliva MMP-8 and MPO, as well as the MMP-8/TIMP-1 ratio. In periodontitis patients, the MMP-8 levels were 31-fold higher in the saliva compared to the serum (1089 vs. 34 ng/ml).

![Graphs showing saliva biomarker levels and ratios](image)

**Figure 8.** The saliva MMP-8, MMP-9, TIMP-1, and MPO concentrations (ng/ml) and the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios in periodontitis, ACS, and ischemic stroke patients. The values were analyzed separately in the Parogene and the Ludwigshafen study populations and are presented as medians and interquartile ranges (IQRs). Statistical significance determined using the nonparametric Mann-Whitney U test. ** refers to p < 0.001 and * refers to p < 0.05.
SMOKING INFLUENCES SERUM BIOMARKER LEVELS

In current smokers, serum MMP-8 and the MMP-8/TIMP-1 ratio were significantly higher compared to that in nonsmokers in the Triangel and Ludwigshafen study populations (Figure 11). In the Triangel study, serum MMP-9 and the MMP-9/TIMP-1 ration were elevated while serum TIMP-1 diminished in smokers. No significant differences according to smoking status (current vs. no current smoking) were found in the Parogene study serum biomarkers using this smoking classification (Figure 9). These analyses included both patients and controls.

Figure 9. Serum MMP-8, MMP-9, TIMP-1, and MPO concentrations (ng/ml) and the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios according to smoking status (nonsmokers vs. current smokers). The values were analyzed separately in the Parogene, Triangel, and Ludwigshafen study populations and presented as medians and interquartile ranges (IQRs). Statistical significance was determined using the nonparametric Mann-Whitney U test. ** refers to p < 0.001 and * refers to p < 0.05.
5.4 SMOKING INFLUENCES SALIVA MMP-9 AND MMP-9/TIMP-1 LEVELS

For the saliva biomarkers only saliva MMP-9 and MMP-9/TIMP-1 measured lower in smokers in the Parogene study (Figure 10). No other differences were observed according to the smoking status using the crude smoking categorization (current smoking vs. nonsmoker) (Figure 10).

![Graphs showing saliva MMP-8, MMP-9, TIMP-1, and MPO concentrations (ng/ml) and the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios according to smoking status (no vs. current smokers). The values were analyzed separately in the Parogene and Ludwigshafen study populations and presented as medians and interquartile ranges (IQR). Statistical significance was determined using the nonparametric Mann-Whitney U test. ** refers to p < 0.001 and * refers to p < 0.05.](image)

Figure 10. The saliva MMP-8, MMP-9, TIMP-1, and MPO concentrations (ng/ml) and the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios according to smoking status (no vs. current smokers). The values were analyzed separately in the Parogene and Ludwigshafen study populations and presented as medians and interquartile ranges (IQR). Statistical significance was determined using the nonparametric Mann-Whitney U test. ** refers to p < 0.001 and * refers to p < 0.05.
5.5 **SMOKING DISTURBS THE DIAGNOSTIC PERFORMANCE OF SERUM MMP-8 AND MMP-9 AND THEIR RATIO TO TIMP-1 IN ACS**

In both the Triangel and Parogene studies, smoking disturbed the diagnostic value obtained through the ROC analysis (Tables 5 and 6). These ROC analyses were performed separately for current smokers and nonsmokers, and thus represent the subgroup analysis.

Table 5. ROC analysis from the Triangel study describing the diagnostic ability of serum MMP-8 and MMP-9 and their molar ratios to TIMP-1 in ACS. Subclasses across the entire Triangel study are presented, and separated into current smokers and nonsmokers.

<table>
<thead>
<tr>
<th>Group</th>
<th>MMP-8</th>
<th>MMP-9</th>
<th>MMP-8/TIMP-1</th>
<th>MMP-9/TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS</td>
<td>0.73</td>
<td>0.75</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>(0.69–0.77)</td>
<td>(0.71–0.79)</td>
<td>(0.67–0.75)</td>
<td>(0.67–0.75)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>0.77</td>
<td>0.73</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>(0.71–0.79)</td>
<td>(0.73–0.82)</td>
<td>(0.69–0.78)</td>
<td>(0.70–0.78)</td>
</tr>
<tr>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>0.62</td>
<td>0.64</td>
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<td>(0.57–0.76)</td>
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<td>Non-smokers</td>
<td>0.71</td>
<td>0.73</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>(0.65–0.78)</td>
<td>(0.67–0.79)</td>
<td>(0.64–0.77)</td>
<td>(0.65–0.78)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AMI</td>
<td>0.76</td>
<td>0.78</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>(0.72–0.80)</td>
<td>(0.74–0.82)</td>
<td>(0.69–0.77)</td>
<td>(0.69–0.77)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>0.77</td>
<td>0.80</td>
<td>0.75</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>(0.72–0.82)</td>
<td>(0.75–0.84)</td>
<td>(0.70–0.80)</td>
<td>(0.71–0.80)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers</td>
<td>0.68</td>
<td>0.71</td>
<td>0.68</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>(0.58–0.79)</td>
<td>(0.62–0.81)</td>
<td>(0.58–0.79)</td>
<td>(0.59–0.79)</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Nonsmokers refers to individuals who do not currently smoke (includes never and former smokers), while smokers refers to individuals who currently smoke.
Table 6. ROC analysis from the Parogene study describing the diagnostic ability of serum MMP-8 and MMP-9 and their molar ratios to TIMP-1 in ACS. Subclasses for the entire dataset are shown as well as separately for current smokers and nonsmokers.

<table>
<thead>
<tr>
<th>Group</th>
<th>MMP-8</th>
<th>MMP-9</th>
<th>MMP-8/ TIMP-1</th>
<th>MMP-9/ TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM (Parogene)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (95% confidence interval (CI)), p value</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS</td>
<td>0.73</td>
<td>0.58</td>
<td>0.74</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>(0.68–0.78)</td>
<td>(0.52–0.64)</td>
<td>(0.69–0.79)</td>
<td>(0.54–0.65)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.04</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>0.75</td>
<td>0.60</td>
<td>0.76</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>(0.70–0.80)</td>
<td>(0.53–0.66)</td>
<td>(0.71–0.81)</td>
<td>(0.55–0.67)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>UAP</td>
<td>NS</td>
<td>NS</td>
<td>0.63</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.50–0.75)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>NS</td>
<td>NS</td>
<td>0.65</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.53–0.77)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AMI</td>
<td>0.76</td>
<td>0.61</td>
<td>0.76</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>(0.71–0.81)</td>
<td>(0.55–0.67)</td>
<td>(0.71–0.81)</td>
<td>(0.56–0.68)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>0.78</td>
<td>0.62</td>
<td>0.78</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>(0.73–0.83)</td>
<td>(0.55–0.68)</td>
<td>(0.73–0.83)</td>
<td>(0.57–0.69)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Nonsmokers refers to individuals who do not currently smoke (includes never and former smokers), while smokers refers to individuals who currently smoke. AUC= area under the curve, ACS= acute coronary syndrome, UAP= unstable angina pectoris, AMI= acute myocardial infarction, NS= not significant.
5.6 THE SENSITIVITY AND SPECIFICITY OF SERUM MMP-8 AND MMP-9 IN ACS

Table 7 summarizes the different sensitivity and specificity pairs showing the cut-off values for serum MMP-8 and MMP-9 obtained from the Parogene study (III) and the Triangel study (I). These cut-off values provided a higher sensitivity, although the specificity decreased. The overall levels of serum MMP-8 and MMP-9 are lower in the Parogene than in the Triangel study. Thus, using the same cut-off values produces multiple false positive (FP) and negative (FN) values. In a multivariate model further adjusted using diabetes, the sensitivity of serum MMP-8 and MMP-9 in ACS in the Triangel study improved, but no effect was not observed in the Parogene study. When adjusting the models further using the crude smoking status (current smoker vs. nonsmoker), no additional improvement was gained (Table 7).

<table>
<thead>
<tr>
<th></th>
<th>SERUM (Parogene)</th>
<th>SERUM (Triangel)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMP-8</td>
<td>MMP-9</td>
</tr>
<tr>
<td><strong>Univariate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>0.73</td>
<td>0.58</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.68–0.78)</td>
<td>(0.52–0.64)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>0.04</td>
</tr>
<tr>
<td>Cut-off²</td>
<td>47.3</td>
<td>170.9</td>
</tr>
<tr>
<td>Sens.</td>
<td>0.65</td>
<td>0.54</td>
</tr>
<tr>
<td>Spec.</td>
<td>0.73</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Multivariate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, sex</td>
<td>AUC</td>
<td>0.74</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.69–0.79)</td>
<td>(0.69–0.77)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Sens.¹</td>
<td>0.65</td>
<td>0.54</td>
</tr>
<tr>
<td>Spec.</td>
<td>0.73</td>
<td>0.58</td>
</tr>
<tr>
<td>Age, sex, diabetes</td>
<td>AUC</td>
<td>0.74</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.69–0.79)</td>
<td>(0.74–0.81)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Sens.¹</td>
<td>0.65</td>
<td>0.55</td>
</tr>
<tr>
<td>Spec.</td>
<td>0.73</td>
<td>0.58</td>
</tr>
<tr>
<td>Age, sex, diabetes,</td>
<td>AUC</td>
<td>0.74</td>
</tr>
<tr>
<td>smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.69–0.79)</td>
<td>(0.73–0.81)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sens.¹</td>
<td>0.64</td>
<td>0.55</td>
</tr>
<tr>
<td>Spec.</td>
<td>0.73</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Smoking refers to nonsmoker vs. current smoker. NS = not significant; ¹Sensitivity according to the specificity determined using the cut-off concentration obtained from the Parogene study, cut-off (μg/L)². Age, sex, diabetes, and smoking covariates in models.
5.7 SERUM MMP-8, MMP-9, AND THEIR RATIO TO TIMP-1 SIGNIFICANTLY ASSOCIATED WITH ACS

The associations of serum MMP-8 and the MMP-8/TIMP-1 ratio to ACS were similar in both the Parogene and Triangel studies. The associations of serum MMP-9 and the MMP-9/TIMP-1 ratio were weaker, albeit statistically significant, in the Parogene study compared to the Triangel study (Table 8). Smoking status (dichotimized as nonsmoker vs. current smoker) emerged as a significant covariate in the logistic regression model calculating the associations of serum MMP-9 and the MMP-9/TIMP-1 ratio to ACS in the Triangel study, but not in the Parogene study (Table 8).

Table 8. The associations of serum biomarkers to ACS in the Parogene (n = 448/481) and the Triangel (n = 597/669) study populations.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Parogene (μg/L)</th>
<th>Triangel (μg/L)</th>
<th>OR (95% CI), p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-8</td>
<td>6.9 (4.2–11.3)</td>
<td>6.8 (4.4–10.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-9</td>
<td>2.3 (1.3–3.8)</td>
<td>10.7 (6.0–19.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>6.6 (4.1–10.6)</td>
<td>5.5 (3.6–8.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-9/TIMP-1</td>
<td>2.4 (1.5–3.9)</td>
<td>6.5 (4.0–10.6)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Age (years) | NS | NS | NS | NS |
Sex (male) | NS | NS | NS | NS |
Diabetes | NS | NS | NS | NS |
Smoking* | NS | NS | NS | NS |

*Smoking refers to nonsmoker vs. current smoker. NS = not significant, OR = odds ratio, CI = confidence interval.
Results

5.8 SALIVA BIOMARKERS SIGNIFICANTLY ASSOCIATED WITH PERIODONTITIS AND SERUM BIOMARKERS WITH ACS

In the Parogene study saliva MMP-8, MMP-9, TIMP-1, and MPO strongly associated with periodontitis, although the association with saliva TIMP-1 was inverse (Figure 11). Saliva MMP-8 exhibited the strongest association with periodontitis. The association between saliva MPO and periodontitis improved in model 5, which was adjusted for age, sex, cardiac status, diabetes, and statins (Figure 11).

By contrast, serum MMP-8, MMP-9, TIMP-1, and MPO strongly associated with ACS, while the association to serum TIMP-1 was inverse. The strongest association to ACS was obtained using serum MPO. Adjusting for age, sex, periodontitis, and diabetes (model 4) improved the associations between the serum MMP-8, MMP-9, and MPO to ACS only slightly (Figure 12).
Figure 11. The associations of saliva biomarkers to periodontitis. The association of saliva biomarkers MMP-8, MMP-9, TIMP-1, and MPO to current periodontitis determined using a logistic regression model and presented as a 10-fold logarithmic scale. Model 1 (M1) is a crude model (n = 481), model 2 (M2) is adjusted for age and sex (n = 481), model 3 (M3) is further adjusted for cardiac status (non-ACS vs. ACS) (n = 453), model 4 (M4) is further adjusted for diabetes (n = 449), and model 5 (M5) is further adjusted for statins (use following hospitalization) (n = 448).
Results

Figure 12. The associations of serum biomarkers to ACS. The association of serum biomarkers MMP-8, MMP-9, TIMP-1, and MPO to ACS determined using a logistic regression model and presented as a 10-fold logarithmic scale. Model 1 (M1) is a crude model (n = 453), model 2 (M2) is adjusted for age and sex (n = 453), model 3 (M3) is further adjusted for periodontal status (no current periodontitis vs. current periodontitis) (n = 453), model 4 (M4) is further adjusted for diabetes (n = 449), and model 5 (M5) is further adjusted for statins (use following hospitalization) (n = 447).
5.9 MMP-8 AND MMP-9 SIGNIFICANTLY CORRELATE WITH EACH OTHER BOTH IN SERUM AND SALIVA

MMP-8 and MMP-9 significantly correlated with each other in the Triangel and the Parogene studies (Figure 13). In serum the Spearman correlation coefficients were 0.668 (p < 0.001) and 0.529 (p < 0.001) in the Triangel and the Parogene studies, respectively. In saliva the correlation between MMP-8 and MMP-9 was 0.611 (p < 0.001).

![Figure 13](image)

**Figure 13.** The Spearman correlation coefficients for MMP-8 and MMP-9 in serum and saliva in the Parogene and the Triangel studies.

5.10 SMOKING DIMENSIONS EXHIBIT DIFFERENT EFFECTS ON SERUM AND SALIVA BIOMARKER LEVELS

Smoking (time since cessation) influenced the saliva MMP-9, serum MMP-8, serum TIMP-1, and serum MPO concentrations in the Parogene study (Table 9). Those who quit smoking less than a year ago exhibited the highest serum MMP-8 and MPO levels, but the lowest serum TIMP-1 concentrations compared to never smokers. By contrast, pack years of smoking affected the saliva MMP-9 and saliva TIMP-1 concentrations. Saliva MMP-9 was lowest in subjects who had smoked more than 20 pack years compared to never smokers, and a significant, decreasing trend was observed across pack-year categories. Those who had smoked more than 20 pack years exhibited significantly higher saliva TIMP-1 values than never smokers. Yet, serum biomarkers did not differ according to pack years (Table 9). When analyzing the biomarker concentrations according to smoking status a bit more indepth using the different smoking dimensions, more information was gained compared to the crude analysis using the nonsmoker vs. current smoker classification (Figures 9 and 10).
Table 9. The saliva and serum biomarker concentrations according to smoking habits and pack years of smoking in the Parogene study.

<table>
<thead>
<tr>
<th>Smoking habit</th>
<th>Saliva, median μg/L (IQR)</th>
<th>Serum, median μg/L (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMP-8</td>
<td>MMP-9</td>
</tr>
<tr>
<td>Quit &gt;1 year ago</td>
<td>779 (252–1277)</td>
<td>200 (65–528)</td>
</tr>
<tr>
<td>Quit &lt;1 year ago</td>
<td>964 (577–1438)</td>
<td>219 (79–540)</td>
</tr>
<tr>
<td>Current</td>
<td>1072 (373–1522)</td>
<td>113 (8–296)</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td>NS</td>
<td>p = 0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Smoking habit</th>
<th>Saliva, median μg/L (IQR)</th>
<th>Serum, median μg/L (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMP-8</td>
<td>MMP-9</td>
</tr>
<tr>
<td>Never</td>
<td>31 (14–73)</td>
<td>149 (78–259)</td>
</tr>
<tr>
<td>Quit &gt;1 year ago</td>
<td>39 (17–89)</td>
<td>163 (91–289)</td>
</tr>
<tr>
<td>Current</td>
<td>35 (17–90)</td>
<td>187 (101–271)</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td>p = 0.006</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pack years of smoking</th>
<th>Saliva, median μg/L (IQR)</th>
<th>Serum, median μg/L (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>917 (397–1390)</td>
<td>242 (104–531)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>822 (334–1306)</td>
<td>164 (58–449)</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td>NS</td>
<td>p = 0.022</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pack years of smoking</th>
<th>Saliva, median μg/L (IQR)</th>
<th>Serum, median μg/L (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>31 (14–73)</td>
<td>149 (78–259)</td>
</tr>
<tr>
<td>1–20</td>
<td>38 (19–86)</td>
<td>163 (98–251)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>41 (14–98)</td>
<td>171 (91–340)</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1Statistically significant difference when comparing never smokers using the nonparametric Mann-Whitney U test (p < 0.05). 2 Statistically significant difference when comparing 0 pack years of smoking using the nonparametric Mann-Whitney U test (p < 0.05). 3The p value between categories tested using the Kruskal-Wallis test. NS = not significant, IQR = interquartile range from the 25th to the 75th percentile.
5.11 SERUM BIOMARKER LEVELS WERE LOWER IN STATIN-TREATED SUBJECTS

The serum MMP-8, MMP-9, and MPO concentrations were significantly lower in patients treated with statin medication than those who were not (Figure 17). Additionally, serum MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios behaved similarly (Figure 14). The saliva biomarkers did not differ according to the use of statins.

Figure 14. The serum and saliva MMP-8, MMP-9, TIMP-1, and MPO concentrations (ng/ml) and the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios according to the use of statins in the Parogene study. Serum values were calculated according to the use of statins before hospitalization, whereas the saliva values were calculated according to the use of statins prescribed during hospitalization. The medians and interquartile ranges (IQRs) are presented. Statistical significance determined using the nonparametric Mann-Whitney U test. ** refers to p < 0.001 and * refers to p < 0.05.
Results

5.12 NUMBER OF CARDIOMETABOLIC MEDICATIONS AND STATINS ASSOCIATED WITH PERIODONTITIS

In univariate and multivariate logistic regression models, the number of cardiometabolic medications significantly associated with periodontitis in the Parogene study (Table 10). In a multivariate model, age and smoking (time since cessation) emerged as significant covariates (Table 10). Additionally, the use of statins significantly associated with periodontitis both in the univariate and multivariate models. In the multivariate model, age, diabetes, and smoking (time since cessation) emerged as significant covariates (Table 10).

Table 10. The association between the number of medications and statins to periodontitis in the Parogene (n = 481) study population.

<table>
<thead>
<tr>
<th></th>
<th>OR (95% CI), p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate</strong></td>
<td></td>
</tr>
<tr>
<td>Number of medications</td>
<td>1.23 (1.09–1.40), 0.001</td>
</tr>
<tr>
<td><strong>Multivariate</strong></td>
<td></td>
</tr>
<tr>
<td>Number of medications</td>
<td>1.19 (1.02–1.40), 0.032</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.04 (1.01–1.06), 0.003</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking*</td>
<td>3.74 (1.82–7.69), &lt;0.001</td>
</tr>
<tr>
<td>CAD status**</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Univariate</strong></td>
<td></td>
</tr>
<tr>
<td>Statins prescribed during hospitalization</td>
<td>2.22 (1.29–3.82), 0.004</td>
</tr>
<tr>
<td><strong>Multivariate</strong></td>
<td></td>
</tr>
<tr>
<td>Statins prescribed during hospitalization</td>
<td>2.00 (1.09–3.68), 0.026</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.04 (1.01–1.06), 0.002</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.69 (1.05–2.73), 0.03</td>
</tr>
<tr>
<td>Smoking*</td>
<td>3.75 (1.83–7.68), &lt;0.001</td>
</tr>
<tr>
<td>CAD status**</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Smoking status refers to never smoker, quit more than 1 year ago, quit less than 1 year ago, or current smoker. The odds ratio (OR) is for the current smoking reference category as a never smoker. **Cardiac status refers to no CAD, stable CAD, ACS, ACS-like, and nonsignificant CAD.
5.13 SERUM TIMP-1 SIGNIFICANTLY ELEVATED DURING THE ACUTE PHASE IN PATIENTS WITH ALL-CAUSE MORTALITY OR CARDIAC DEATH DURING FOLLOW-UP

The serum TIMP-1 concentrations measured during the acute phase were significantly higher in subjects with all-cause mortality or cardiac death both in the Parogene and the Triangel studies (Figures 15A and 15B). Furthermore, in the Triangel study, the serum MMP-8 and MMP-9 levels were significantly elevated in patients who died due to cardiac-related causes during the follow-up period (Figure 15B). However, in the Parogene study, the acute-phase serum MMP-8, MMP-9, or MPO levels did not differ significantly according to mortality (Figure 15A).

**Figure 15A.** Acute-phase serum MMP-8, MMP-9, TIMP-1, and MPO concentrations (ng/ml) by mortality in the Parogene study. The end points were no death (n = 427), death due to any reason (n = 54), and cardiac-related death (n = 34) during the follow-up period. The medians and interquartile ranges (IQRs) are presented. Statistical significance determined using the nonparametric Mann-Whitney U test and using the no death group as the comparison value. * refers to p < 0.001.

**Figure 15B.** Acute-phase serum MMP-8, MMP-9, and TIMP-1 concentrations (ng/ml) according to cardiac death in the Triangel study. The end points were no death (n = 589) and cardiac-related death (n = 74) during the follow-up period. The medians and interquartile ranges (IQRs) are presented. Statistical significance determined using the nonparametric Mann-Whitney U test and using the no death group as the comparison group. * refers to p < 0.001.
**5.14 ASSOCIATION OF CARDIOMETABOLIC MEDICATIONS TO CARDIAC DISEASE AND MORTALITY**

Serum MMP-8 and TIMP-1 associated with an increased risk for death due to any cause as well as cardiac death in the multivariate Cox regression models (Table 11). After further adjusting the model for the use of statins, the associations to both death end points increased (Table 11).

**Table 11.** The associations of serum biomarkers to all-cause and cardiac death in the Parogene study group as well as the effect of statins.

<table>
<thead>
<tr>
<th></th>
<th>MMP-8 Hazard ratio (HR) (95% CI), p value</th>
<th>MMP-9 Hazard ratio (HR) (95% CI), p value</th>
<th>TIMP-1 Hazard ratio (HR) (95% CI), p value</th>
<th>MPO Hazard ratio (HR) (95% CI), p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All-cause death</strong> Model 1</td>
<td>1.85 (1.0–3.4), 0.05</td>
<td>NS</td>
<td>100.9 (13–763), NS</td>
<td>NS</td>
</tr>
<tr>
<td>Model 2</td>
<td><strong>1.98 (1.1–3.6), 0.025</strong></td>
<td>NS</td>
<td>86.5 (11–697), NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Cardiac death</strong> Model 1</td>
<td>2.18 (1.0–4.7), 0.045</td>
<td>NS</td>
<td>204.8 (18–2394), NS</td>
<td>NS</td>
</tr>
<tr>
<td>Model 2</td>
<td><strong>2.34 (1.1–5.0), 0.026</strong></td>
<td>NS</td>
<td>177.4 (14–2249), NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Model 1 adjusted for age, sex, cardiac status, and diabetes (n = 449). Model 2 further adjusted for statins prescribed during hospitalization (n = 448). The hazard ratios (HRs) for the acute-phase serum MMP-8, MMP-9, TIMP-1, and MPO concentrations (per unit increase) calculated using the Cox regression model, where the end points are death due to any cause or due to cardiac death during the follow-up period.

**5.15 PROGNOSTIC VALUE OF ACUTE- AND RECOVERY-PHASE SERUM MMP-8, MMP-9, AND TIMP-1 FOR MACE**

An elevated acute-phase serum MMP-9 level associated with an increased risk for a fatal major adverse cardiac event (MACE), whereas an elevated serum MMP-8 level and an MMP-8/TIMP-1 ratio during the acute phase weakened these associations with a non-fatal MACE in the Triangel study (Figure 16). If the serum MMP-9 concentrations or the MMP-9/TIMP-1 ratio persisted at a high level during the recovery phase, the risk for MACE, particularly a nonfatal MACE, increased. The largest decrease in the serum MMP-8 and MMP-9 levels between the acute and recovery phases (ΔMMP-8 and ΔMMP-9) protected individuals from MACE, particularly from a nonfatal MACE (Figure 16).
In addition, an elevated acute-phase serum TIMP-1 level associated with an increased risk for MACE, particularly a fatal MACE, during the follow-up period. Furthermore, an elevated recovery-phase serum TIMP-1 associated with an increased risk for a fatal MACE and diminished the risk for a nonfatal MACE (Figure 16).

To summarize, elevated serum MMP-9 and TIMP-1 concentrations during the acute and recovery phases associated with MACE and its subclasses. The largest decrease in MMP-8 and MMP-9 (Δ values) protected individuals from MACE, particularly a nonfatal MACE.
Results

Figure 16. The hazard ratios (HRs) for a major adverse cardiac event (MACE) and the subgroups of a nonfatal MACE (new ACS) or a fatal MACE (cardiac death) according to the serum MMP-8, MMP-9, TIMP-1 concentrations, the MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios, and Δ values (acute–recovery) in the 6-year follow-up period in the Triangel study. HRs calculated using the Cox regression model adjusted for age and sex.
6 SUMMARY AND DISCUSSION

In this PhD study serum and saliva biomarkers for periodontal and atherosclerotic CVDs were investigated. Serum biomarkers appear suitable for differentiating CVDs from controls, whereas saliva biomarkers differentiate periodontal disease. Additionally, serum biomarkers may serve as predictive markers for future cardiac events or mortality. Smoking, periodontal status, cardiac disease, the number of cardiometabolic medications, and statins emerged as factors affecting both local and systemic biomarkers, and these factors were investigated within the framework of this study.

6.1 SERUM BIOMARKERS VALUABLE IN ACS AND ISCHEMIC STROKE

The results from this study indicate that the serum biomarkers were significantly elevated in CVD, both for ACS and ischemic stroke. Serum MMP-8, the MMP-8/TIMP-1 ratio, and MPO were significantly elevated in ACS and ischemic stroke, while serum MMP-9 and the MMP-9/TIMP-1 ratio were significantly elevated in ACS. Furthermore, serum MMP-8 and MMP-9 and their ratios to TIMP-1, as well as serum MPO, strongly associated with ACS. Our findings support earlier studies in which systemically elevated MMP-8 and MMP-9 associated with CAD, ACS, and ischemic stroke (Kato et al. 2005, Pussinen et al. 2013, Momiyama et al. 2010, Palm et al. 2018, Kalela et al. 2002, Fukuda et al. 2006, Kai et al. 1998, Turu et al. 2006,). Additionally, an elevated serum MPO associated with ischemic stroke (Palm et al. 2018, Tay et al. 2015). However, in the Parogene study, the serum TIMP-1 concentrations alone did not lead to AUC values in ACS diagnostics, a finding that differs from previous findings from the Triangel study and in other populations in which systemic TIMP-1 differentiated ACS patients from healthy controls (Pussinen et al. 2013, Inokubo et al. 2001, Cavusoglu et al. 2006, Noji et al. 2001, Pradhan-Palikhe et al. 2010). The low discrimination power of serum TIMP-1 in the Parogene study may be due to the reference group, which consisted not of healthy controls, but symptomatic non-ACS patients.

6.2 SALIVA BIOMARKERS DIFFERENTIATE PERIODONTITIS, BUT NOT CVD

The saliva MMP-8, MMP-9, and MPO concentrations as well as the saliva MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios were significantly higher among periodontitis patients when compared to the no periodontitis control
Summary and discussion

Group. Yet, saliva TIMP-1 was significantly diminished among periodontitis patients. While all saliva biomarkers—that is, MMP-8, MMP-9, TIMP-1, and MPO—associated significantly with periodontitis, the association between saliva TIMP-1 was inverse. Moreover, saliva MMP-8 most strongly associated with periodontitis, a result consistent with earlier studies (Sorsa et al. 2016, Miller et al. 2006, Gursoy et al. 2010, Zhang et al. 2018). Additionally, elevated oral MMP-9 and MPO levels have been detected in periodontitis patients (Gursoy et al. 2013, Nizam et al. 2014, Marcaccini et al. 2010).

Ischemic stroke patients more often had missing teeth and edentulism, indicating end-stage periodontitis, whereas controls had higher saliva MMP-8, MPO, IL-1β, and periopathogenic bacterial levels, indicative of current periodontal disease. These findings demonstrate that local saliva biomarkers did not reflect systemic CVD status, but merely reflected overall oral-health status. Unfortunately, in this study, we did not have clinical oral and periodontal examination data available. Thus, saliva biomarkers were used to estimate the risk of having periodontitis as described earlier (Gursoy et al. 2011).

Saliva research has received increasing attention, since collecting saliva is noninvasive, easy, and cost-effective, and saliva has been investigated as a diagnostic tool in dentistry, medicine, and pharmacotherapy (Kaczor-Urbanowicz et al. 2017). Furthermore, saliva biomarkers have been investigated in cardiac diagnostics as a panel of combined biomarkers during the early stage of AMI (Floriano et al. 2009) or separately as saliva MMP-9, MPO, CRP, and IL-1β as biomarkers for AMI (Christodoulides et al. 2012). However, saliva rather broadly reflects the oral-health status since it contains many inhibitors and periodontitis-derived enzymes, thus complicating its use in systemic disease diagnostics (Miller et al. 2010). Our findings in the Parogene and Ludwigshafen studies in particular reflect this complication.

In this PhD study, MMP-8 and MMP-9 correlated significantly with each other both in the serum and saliva. Similar significant and strong correlations between plasma MMP-8 and MMP-9 were also previously reported (Turu et al. 2006). In the Parogene study, MMP-8, MMP-9, and MPO correlated with each other when measured in either the serum or saliva, although the serum and saliva biomarkers did not correlate with each other, indicating that the enzymes are locally produced. Another explanation may lie in the time gap between the serum and saliva collections, which varied from 37 to 224 days. Thus, serum, drawn immediately following angiography, clearly reflects an acute-phase response (ACS and ACS-like nonsignificant CAD), whereas during saliva collection the acute-phase response has ended, possibly affecting the serum and saliva associations.
6.3 SMOKING COMPLICATES THE USEFULNESS OF BIOMARKERS

Smoking is a complex factor affecting and modulating inflammation and serving as a strong risk factor for both periodontitis and CVDs. Smoking has three dimensions: duration, quantity, and time since cessation (Liu et al. 2016). Furthermore, smoking systemically and locally affects the oral cavity. Earlier studies found both elevated and decreased levels of oral and systemic MMP-8, MMP-9, TIMP-1, and MPO due to smoking exposure.

In this PhD study, current smokers exhibited elevated serum MMP-8 and MMP-9 levels and MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios when compared to nonsmokers. In addition, smoking impeded the diagnostic value of serum MMP-8, MMP-9, and their ratio to TIMP-1 in ACS, although a multivariate model further adjusted for smoking (determined comparing nonsmokers vs. current smokers) did not improve the C-statistics diagnostics value. ROC subgroup analyses revealed a smaller group size, possibly affecting the results.

Yet, current smokers had lower saliva MMP-9 and MMP-9/TIMP-1 ratio values compared to nonsmokers. When analyzing different smoking dimensions, a smoking habit (time since cessation) emerged with the strongest effect on the saliva MMP-9, serum MMP-8, serum TIMP-1, and serum MPO concentrations. Pack years, however, affected the saliva MMP-9 and TIMP-1 concentrations. Furthermore, pack years of smoking affected the saliva MMP-8. More specifically, when adjusting the logistic regression model further using pack years, the association of saliva MMP-8 to periodontitis improved.

Smoking diminishes microcirculation in the periodontium, possibly concealing signs of clinical disease (Hayman et al. 2011). Moreover, the effects of smoking on the amount and secretion of GCF (Ustün et al. 2007, Morozumi et al. 2004) may also affect the amount of MMP in the saliva. However, the most considerable effect of smoking on salivary MMP is probably mediated by the immunosuppressant effects of smoking, such as an impaired neutrophil functioning (Söder et al. 2002) and a decrease in the levels of several cytokines (Tymkiw et al. 2011).

Smoking stands as a variable that remains hard to accurately estimate in epidemiological studies. The objective amount, duration, and time since cessation are impossible to capture since self-reported smoking habits are commonly biased. Cotinine, a metabolite of nicotine, can be regarded as a biomarker of smoking or nicotine intake, with a half-life of 15 to 20 h (Benowitz et al. 2009). Measuring the saliva or serum cotinine levels would have been more accurate and objective in determining smoking exposure. But
Summary and discussion

this method also carries challenges, such as determining reliable cut-off values, interpreting long-term exposure to cigarette smoke, and identifying false positives due to nicotine replacement therapies (Kim 2016). Additionally, cotinine levels depend on genetics and differences in metabolic pathways; thus, the use of cotinine as a biomarker of nicotine intake should be carefully considered (Ware et al. 2016).

The local and systemic effects of smoking and their long-term impact after cessation varies, thereby necessitating investigation of the different smoking dimensions and their effects on biomarkers. While the risk of periodontitis decreases with time since smoking cessation (as early as 1 year after cessation), smoking presents a persistent and increased risk for tooth loss at least 10 years following smoking cessation (Dietrich et al. 2007). Other studies observed a similar declining trend as well as the beneficial effect of cessation on the risk of tooth loss, while researchers speculated that it may take decades—possibly as long as 30 years—to return to the level of never smokers (Krall et al. 1997, Arora et al. 2010, Krall et al. 2006). Other nicotine and cigarette products also affect periodontal health, while data from these products remain more limited than tobacco cigarettes. For example, pipe and cigar smoking have been associated with an increased risk of tooth loss, similar to the risk for cigarette smokers (Krall et al. 1999, Dietrich et al. 2007). Additionally, smokeless tobacco has been associated with periodontitis (Fisher et al. 2005).

6.4 OTHER FACTORS AFFECTING BIOMARKERS

Many systemic diseases, human behaviors, genetic factors, medications, age, sex, hormones, pregnancy, and the individual’s ethnic background may influence biomarkers. Diabetes has been associated with elevated systemic MMP-8, MMP-9, and TIMP-1 levels (de Morais et al. 2018, Sundström et al. 2004b, Ebihara et al. 1998, Derosa et al. 2007, Signorelli et al. 2005, Derosa et al. 2007, Lee et al. 2005). Age-related differences have been observed in plasma MMP-9, TIMP-1 and the MMP-9/TIMP-1 ratio (Bonnema et al. 2007, Sundström et al. 2004b), while circulating MMP-9 levels differed according to ethnicity (Taybjee et al. 2005).

In the Parogene study, periodontitis interfered with the use of serum MMP-9 in ACS diagnostics, whereas the cardiac status affected the usefulness of saliva TIMP-1 in periodontitis. Furthermore, the cardiac status influenced saliva MPO sensitivity in the diagnosis of periodontitis. Earlier studies demonstrated the beneficial effect of periodontal treatment on systemic inflammatory markers and MMP-9 (Behle et al. 2009, Marcaccini et al. 2009), supporting the effect of periodontitis on systemic inflammation and circulating biomarkers. Periodontitis and CVD associate with one another (Lockhart et al. 2009).
2012), thus reasonably indicating the influence of periodontitis on cardiac diagnostics and vice versa.

6.5 STATINS AND OTHER CARDIOMETABOLIC MEDICATIONS AFFECT CVD, PERIODONTITIS, AND THEIR RELATED BIOMARKERS

Serum MMP-8, MMP-9, and MPO, as well as the MMP-8/TIMP-1 and MMP-9/TIMP-1 ratio emerged as significantly lower in statin-treated subjects compared to subjects not receiving statin medication. Our findings support earlier studies, where statins reduced systemic MMP-8, MMP-9, TIMP-1, and MPO and the MMP-8/TIMP-1 ratio describing the pleiotropic effect of statins, that is, reducing systemic inflammation (Kadoglou et al. 2014, Koh et al. 2002, Lubos et al. 2006, Mayyas et al. 2018). The saliva biomarker concentrations, however, did not differ according to statin use.

In addition, the number of cardiometabolic medications associated with an increased risk for periodontitis, while the use of statins associated with periodontitis. The use of cardiometabolic medications probably reflects an overall worse systemic health status, possibly also affecting periodontal health. Thus, these associations may be explained by the subjects’ worse conditions. Furthermore, multiple medications associate with hyposalivation, which itself increases the risk for oral diseases such as caries, yeast infections, and periodontitis.

6.6 THE PREDICTIVE VALUE OF BIOMARKERS

Systemically elevated MMP-8, MMP-9, TIMP-1, and MPO have been associated with an increased risk for a cardiac event and mortality (Tuomainen et al. 2007, Peeters et al. 2011, Kormi et al. 2017, Blankenberg et al. 2003, Cavusoglu et al. 2006, Hansson et al. 2011, West et al. 2008, Lubos et al. 2006, Pussinen et al. 2013, Frantz et al. 2008, Heslop et al. 2010, Tay et al. 2015). Similar findings were also obtained in the Triangel and Parogene studies, where elevated serum MMP-8, MMP-9, and TIMP-1 associated with a CVD event or death. Interestingly, the largest decrease in MMP-8 and MMP-9 (Δ values) during the recovery period protected individuals from MACE, particularly nonfatal MACE in the Triangel study.

Within the framework of this thesis, we were only able to investigate the prognostic value of the serum biomarkers in CVD. In the future, researchers should collect longitudinal data to also consider saliva biomarkers and periodontitis. Currently, our saliva analysis using periodontitis is cross-
sectional. Thus, we cannot estimate the progression of periodontal disease, which would be interesting. Evaluating the individual as well as site-specific periodontal disease activity and attempting to predict disease progression remains of great interest in periodontal research. Currently, few datasets are available which include oral biomarkers as predictors of periodontal disease. Yet, high- and low-response patterns relying on the MMP-8 levels measured from GCF were separately identified among smokers and nonsmokers (Leppilahti et al. 2015). The clinical parameters in periodontal diagnosis, such as PPD, BOP, and CAL together with radiographs, indicate current disease and a cumulative disease history, yet remain poor predictors of the future course of disease. Therefore, individual risk assessment is supplemented with systemic and local risk factors (Papapanou et al. 2018, Caton et al. 2018).

6.7 THE CHALLENGES IN DETERMINING UNIVERSAL CUT-OFF VALUES

Several questions emerge when determining the universal cut-off values for biomarkers. In clinical practice either specificity or sensitivity should be emphasized more depending upon the situation. Factors to consider as more important include the following: the high proportion of true negatives (higher specificity) or the high proportion of true positives (higher sensitivity). When considering CAD, preference is placed on a higher cut-off value (emphasizing specificity) to distinguish true ACS cases from other acute events with similar clinical symptoms. When considering periodontitis, a lower threshold (emphasizing sensitivity) is preferable in order to identify subjects requiring more periodontal guidance or treatment.

Periodontitis activity fluctuates and periods of exacerbation and stability alter with one another (Socransky et al. 1984, Graves et al. 2011). A certain level of MMPs is necessary for normal tissue homeostasis (Kuula et al. 2009, Hernandez et al. 2011). In periodontitis patients without other risk factors, decreased oral MMP-8 levels may indicate remission, although in smokers, for example, a decreased MMP-8 level may indicate a suppressed host response (Leppilahti et al. 2015). We can assume that each patient possesses an individual MMP profile depending on the host response, the inflammatory response, genetics, epigenetics, metabolic pathways, other risk or modulating factors, particularly smoking status and history, site-specific factors, and the microbial burden. Thus, the biomarker results should be interpreted with caution.

In the Parogene study, we determined the cut-off values that produced the highest sensitivity and specificity while simultaneously providing the most objective result without emphasizing either the sensitivity or specificity in
particular. When replicating the same thresholds in the Triangel study, we encountered a challenge: the same cut-offs produced a higher sensitivity, but a lower specificity. The median concentrations of serum MMP-8 and MMP-9 were lower in the Parogene study than in the Triangel study, whereby determining reliable cut-offs that distinguished ACS patients from the reference group for both studies remained challenging. Additionally, the median concentrations of serum MMP-8, MMP-9, and MPO in the Ludwigshafen study emerged as the lowest (both in the ischemic stroke patients and controls) when compared to the Parogene or the Triangel studies. Several reasons may explain why the median levels of the biomarkers differed across studies. The Parogene, Ludwigshafen, and Triangel studies were collected at different times, and some of the laboratory determinations were performed on samples frozen long before analysis. However, we found no significant effect of the storage time on the biomarker levels using our in-house laboratory quality control mechanisms (AlfaKry 2014). Another explanation might lie in the variation between commercial ELISA kits, since different lots were used in each study. No blind controls used by clinical laboratories (that is, controls performed blindly in different laboratories) were available for these commercial kits, which could be used for quality control purposes. In addition, laboratory processing may cause errors, such as errors related to sample processing. However, the samples for these specific studies were processed similarly and statistically significant differences between cases and their selected controls were calculated. Thus, these differences can be reliably examined and their clinical relevance discussed.

**Active and activatable gelatinases**

In a representative gelatin-zymography analysis, we randomly selected a subsample of patients suffering MACE and their controls from the Triangel population. Cases with a fatal MACE had the highest activatable MMP-9 level during the acute phase, providing a new perspective. The MMP-9 activation potential may offer new insights and warrants further study.

Gelatin-zymography is a semiquantitative method and the total amount of active MMP-9 cannot be estimated since we only have information on the portion of MMP-9 activated via proteolysis (Kupai et al. 2010). We can assume that part of the MMP-9 activation occurs through an alternative activation without a change in the molecular weight (Bannikov et al. 2002). Furthermore, the functional activity of MMP-9 under physiological conditions cannot be addressed, since SDS interrupts the interactions between MMP-9 and TIMP-1 (Ikeda et al. 2000). Our zymography results show that naturally active MMP-9 is detectable in our samples, and when in circulation active MMP-9 is primarily inhibited by TIMP-1, serum α2-macroglobulin, and other inhibitors (Brew et al. 2000, Yabluchanskiy et al. 2013). However, we can
hypothesize that zymography produces information on active and activatable MMP-9 and compare the results with the control values obtained similarly.

6.8 LIMITATIONS AND STRENGTHS OF THE STUDY

Limitations of the study
There are several limitations concerning this PhD thesis and its substudies. None of the study settings offered the possibility to evaluate the biomarker levels prior to an acute event. Thus, we cannot be certain about the origin of the systemic biomarkers following an acute CVD event. The Triangel study population was collected in 1999 to 2002 using the prevailing diagnostic criteria regarding ACS. Several diagnostic and therapeutic changes subsequently occurred, possibly affecting the results. Currently, high-sensitivity TnT is utilized in clinical practice, altering the ACS diagnostic criteria. However, the changes in the serum biomarkers were so high among ACS patients, both in the AMI and UAP subgroups, that it is unlikely that changes in the general classifications would have dramatically changed our primary findings. Furthermore, the Triangel study only contained information about “lipid-lowering medications”. Thus the effect of statins could not be addressed here. The use of statins has increased following the collection of the Triangel data. Smoking status among the Triangel study subjects relied on self-reports, and could not be collected from all participants. Hence, inaccuracies might have occurred. Furthermore, only information about current smoking was collected. Thus, the effect of pack years or smoking cessation could not be investigated.

The Ludwigshafen data do not include clinical oral examination data. Thus, we cannot evaluate the rate of periodontitis among patients and the association between periodontitis and ischemic stroke. Moreover, we were only able to estimate the periodontal state using the saliva biomarkers and the CRS score to anticipate the risk of having periodontitis, as well as by using self-reported information about missing teeth and if a dentist had diagnosed periodontitis. Typically, patients remain unaware of their periodontal status, because periodontal diseases can develop without symptoms. Additionally, we do not have follow-up data. Thus, this study design is cross-sectional, and we do not have samples from the same patients at different time points allowing us to perform intrapersonal analyses and to evaluate the course of biomarker levels following an acute event.

The Parogene study also carries several limitations. For instance, the Parogene cohort consists of relatively old and diseased individuals, all with an indication of coronary angiography. Thus, the reference group did not consist of healthy
controls, possibly affecting our results. Furthermore, many of the Parogene subjects were heavily medicated, reflecting their poor health status. In our study, a patient was classified as having periodontitis if both ABL (mild to severe) and PPD ≥4 mm in ≥4 sites were observed. New criteria for periodontitis were recently published (Papapanou et al. 2018). According to these criteria, a patient receives a diagnosis of periodontitis if (CAL) is interdentally detected in ≥2 nonadjacent teeth or buccally or orally ≥3-mm CAL is detected in ≥2 teeth without nonperiodontitis-related causes; this diagnosis is supplemented by the stage and grade of the disease (Papapanou et al. 2018, Caton et al. 2018). Overall, the Parogene patients are rather periodontally diseased. The control group for periodontitis patients also contains gingivitis patients, possibly affecting the discrimination power of the saliva biomarkers. For example, saliva MMP-8 differentiates gingivitis patients from periodontally healthy patients (Noack et al. 2017). Hence, our control group may have exhibited elevated enzyme levels caused by gingivitis. However, our aim was to differentiate periodontitis patients in which these biomarkers performed rather well. In the future, further studies should investigate how the saliva biomarkers (MMP-8, MMP-9, TIMP-1, and MPO) would differentiate between various stages or even the progression rate of periodontal disease.

To summarize, our periodontal diagnosis and reference group as well as the study design may have affected the results obtained from the saliva biomarkers. The Parogene study is primarily cross-sectional, and samples from the same subjects at several time points were not available. Thus, intrapersonal analyses could not be performed. Furthermore, information related to smoking habits and exposure contained inaccuracies. Specifically, not all subjects reported the amount they smoked, whereby information related to pack years for some subjects remains unknown.

**Strengths of the study**

This thesis and its substudies enabled a comparison of serum and saliva biomarkers in different populations, and aimed to reveal the association between periodontitis and CVD. The Parogene and Triangel studies were relatively large cohorts of subjects in which we could explore the association between the serum biomarkers and ACS. Furthermore, the Parogene study included the detailed clinical oral health status of patients with angiographically verified CAD allowing investigation of both the oral and cardiac biomarkers and their confounders. The Ludwigshafen study offered an interesting setting in which to compare the serum and saliva biomarkers in ischemic stroke patients and healthy controls. In the Parogene study, we could investigate both the time since cessation and pack years of smoking, affording
us the opportunity to investigate the dimensions of smoking more profoundly. In the Triangel study, we investigated the serum biomarker levels during both the acute and recovery phase and, therefore, also included the Δ values in the analysis. Additionally, we investigated activatable MMP-9 and its relation to CVD end points. In the results section of this thesis, we could also compare the results across studies.
7 CONCLUSIONS AND FUTURE PROSPECTS

The predictive and diagnostic tests for periodontal and cardiovascular diseases are welcome in clinical practice, although solid evidence about factors affecting these biomarkers are needed before introducing them for large-scale use. This PhD project expanded our knowledge of the link between oral health and CVD by investigating the biomarkers of both diseases. Since periodontitis associates with an increased CVD risk, novel tools for diagnosing periodontitis and CVD remain valuable for exploration. Identifying easy, quick, and reliable markers remains urgent. Biomarkers reflecting the periodontal state can be utilized both by dentists and other healthcare professionals in medical centers. Biomarkers for the diagnosis and prognostics of fatal CVD would more precisely identify potential major cardiovascular events (MACEs). As such, biomarkers could be used to differentiate patients otherwise indistinguishable using clinical criteria alone. In addition, primary and secondary prevention of these diseases would prove economical, as well as highly valuable from patients’ perspectives and considering their quality of life.

The data from this PhD study indicate that serum biomarkers are suitable for distinguishing CVD, whereas the saliva biomarkers describe the local periodontal status. The serum biomarkers may also hold predictive value concerning future cardiac events or mortality. In addition, smoking represents a significant factor affecting the course of disease for both periodontitis and CVD. Smoking also disturbs the biomarker diagnostics of these diseases.

The key findings from the thesis are as follows:

I. Serum biomarkers associated strongly with ACS and ischemic stroke.
II. Saliva biomarkers associated significantly with periodontitis.
III. Smoking affected the serum and saliva biomarkers, although current smoking, pack years, or time since cessation affected these biomarkers in different ways.
IV. Periodontitis decreased the diagnostic value of serum MMP-9 in ACS, whereas the cardiac status complicated the use of saliva TIMP-1 in periodontitis.
V. An elevated acute- and recovery-phase serum MMP-9 and TIMP-1 associated with MACE, and the largest decrease in the MMP-8 and MMP-9 (Δ values) levels indicated a protective value against MACE, particularly for nonfatal MACE.
Conclusions and future prospects

In general, the challenge with any biomarker is that biomarkers do not always reflect the desired phenotype. Individual differences in genetics, epigenetics, and metabolic pathways may influence the reliability of the biomarkers. Furthermore, the biological processes and pathways behind diseases should be well-characterized in order to target the right biomarkers. For example, MMPs are also important during recovery related to angiogenesis and healing. Thus, they play a remarkable role in normal recuperative processes. In the future, more personalized diagnostics and prognostics for oral and systemic diseases should be used.

Future research should investigate those factors affecting these biomarkers. Large-scale longitudinal and multicenter studies with data from clinical and radiographical examinations, as well as microbiome, genomics, proteomics, and metabolomics analyses integrated with one another would provide new information about the associations between periodontal and cardiovascular diseases and their related biomarkers. In the future, baseline samples from healthy volunteers such as blood donors could be collected and analyzed. Blood donors typically donate several times during their lives, rendering intrapersonal analyses possible. Medical record data and information about disease end points could be collected from registries, which are well-maintained in Finland. The attitude of the Finnish people towards scientific studies remains positive, and, thus, reliable register studies as well as biobank studies are possible among a Finnish population. Additionally, fingertip blood samples from diabetic patients could be collected quite easily and their end points and biomarkers could be analyzed. Saliva is also an easy fluid to collect even at home. Thus, saliva sampling could be easily added to study protocols. Currently, all dental record data are collected in the national Kanta registry, rendering oral examination data potentially useful. Digitalization and well-organized registries as well as biobanks create a fruitful environment for new research aimed at investigating the link between oral and systemic diseases and their related biomarkers.
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