SALIVARY BIOMARKERS

- Diagnostic potential in oral and systemic diseases in epidemiological surveys

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By

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This thesis is dedicated to:

My mother

“After climbing a great hill, one only finds that there are many more hills to climb”

- Nelson Rolihlahla Mandela

(1918 – 2013)
ABSTRACT

Human saliva is a fluid with many biological functions, and essential for the maintenance of oral health. Several studies report local and systemic biomarkers appearing in saliva, including electrolytes, blood products, enzymes and tissue destruction molecules, inflammatory markers as well as proteins putatively associated with different diseases. However, the clinical utility of salivary diagnostics for the assessment of oral and systemic disease remains elusive. The general aim of this project was to investigate the utility of salivary biomarkers for diagnostic potential of oral- and systemic diseases in large populations.

This thesis consists of two different projects:

(i). Skåne cohort - A cross-sectional study (Studies I and II): 451 individuals were randomly selected and enrolled for these investigations, including 51% women. All participants were asked to complete a questionnaire, a medical history was taken, a clinical examination was made and stimulated saliva samples were collected.

(ii). PAROKRANK sub-cohort (Periodontal disease and the relation to myocardial infarction) (Studies III and IV): A case-control study comprising 400 subjects. In total 200 consecutive patients with a first acute myocardial infarction (AMI) admitted to coronary care units in Sweden from May 2010 to December 2011, and 200 controls without previous AMI, matched for age, gender, residential area were included during the same time period. Dental examinations were performed, blood and stimulated saliva samples were collected eight to ten weeks after the myocardial infarction (MI). Matched controls were examined within one to two weeks after the MI patients.

Study I: The aim of this study was to investigate if selected salivary biomarkers could be used for epidemiological studies for detection of periodontitis. Our findings showed that patients with severe periodontitis had elevated salivary concentrations of interleukin (IL) -1β (IL-1β) and matrix metalloproteinase (MMP) -8 (MMP-8), as well as and increased ratio of MMP-8/ tissue inhibitor of metalloproteinase-1 (TIMP-1).

Study II: The aim of this study was to investigate if certain salivary biomarkers could be used for detection of common systemic inflammatory diseases. The results of our study showed that salivary IL-8 concentrations were higher in patients with bowel disease and subjects who had experience of tumor diseases. MMP-8 levels were elevated in saliva from patients with diabetes, muscle and joint diseases or previously had undergone cardiac surgery.

Study III: The aim of this study was to investigate whether salivary concentrations of selected cardiovascular biomarkers could be used to identify patients with a previous MI and whether such markers, in plasma and saliva, were related to periodontal status. There was no difference between participants with or without MI in regards of N-terminal prohormone of brain natriuretic peptide (NT-pro BNP) and growth differentiation factor-15 (GDF-15) levels in saliva. However, cystatin C, NT-pro BNP and GDC-15 levels were higher in MI patients with other co-morbidities. The levels of cystatin C were lower in saliva from patients with MI. GDF-15 levels correlated with periodontal status in both groups. Further, there was no correlation between plasma and saliva levels.
Study IV: The aim of this study was to explore the levels of the inflammatory markers, MMP-8, MMP-9, TIMP-1 and myeloperoxidase (MPO) in saliva with regards to previous MI and periodontal disease. The analyzed biomarkers correlated significantly with each other and most of the periodontal parameters in both study groups. Salivary MMP-8 and MPO levels were significantly higher in non-MI subjects.

In conclusion, the findings in this project indicates that certain salivary biomarkers have the potential to be used for screening purposes in epidemiological studies related to both oral and systemic diseases. In addition, the selected salivary biomarkers in our studies could be seen as markers for increased local and systemic inflammation.

Key words: Interleukin-1β, -6 and -8, Cystatin C, Growth differentiation factor-15, Matrix metalloproteinases-8 and -9, Myeloperoxidase, Myocardial infarction, Periodontal disease, Plasma, Saliva, Tissue inhibitor of metalloproteinase-1
LIST OF SCIENTIFIC PAPERS

This thesis is based upon the following papers, which will be referred to in the text by their Roman numerals:


III. Nilminie Rathnayake, Björn Klinge, Anna Norhammar, Kåre Buhlin, Taina Tervahartiala, Christian Löwbeer, Timo Sorsa, Anders Gustafsson. Saliva and plasma levels of cardiac related biomarkers in patients with a previous acute myocardial infarction – A subgroup report from the case-control PAROKRANK study (Periodontal disease and the relation to myocardial infarction study). (Submitted)

IV. Nilminie Rathnayake, Anders Gustafsson, Anna Norhammar, Björn Klinge, Taina Tervahartiala, Timo Sorsa. Associations between Salivary Matrix Metalloproteinase-8 and -9 and Myeloperoxidase in myocardial infarction patients with or without periodontal disease – A subgroup report from the case-control PAROKRANK study (Periodontal disease and the relation to myocardial infarction study). (Submitted)
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACS</td>
<td>Acute coronary syndromes</td>
</tr>
<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme inhibitors</td>
</tr>
<tr>
<td>AMPs</td>
<td>Antimicrobial Peptides</td>
</tr>
<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>CK-MB</td>
<td>Creatine kinase-MB</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix proteins</td>
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<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
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<tr>
<td>GDF-15</td>
<td>Growth differentiation factor-15</td>
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<tr>
<td>IFMA</td>
<td>Immunofluorometric assay</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>IL-1β</td>
<td>Interleukin-1β</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<td>IL-8</td>
<td>Interleukin-8</td>
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<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<td>MMP-8</td>
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<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinases-9</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>N-terminal prohormone of brain natriuretic peptide</td>
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<tr>
<td>PG</td>
<td>Parotid glands</td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclears leukocytes</td>
</tr>
<tr>
<td>PPD</td>
<td>Probing pocket depth</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SLG</td>
<td>Sublingual gland</td>
</tr>
<tr>
<td>SMG</td>
<td>Submandibular glands</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue inhibitors of matrix metalloproteinases-1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-α</td>
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<td>WHO</td>
<td>World Health Organization</td>
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INTRODUCTION

HUMAN SALIVA

Whole saliva is a unique biological fluid and it has been described as the “mirror of the body”. Saliva is composed mainly of water (98%) and about 2% other compounds, such as minerals, electrolytes, hormones, antibacterial compounds, mucins, various enzymes, immunoglobulins and cytokines (1). Primary saliva is produced by major and minor salivary glands essentially. Salivary secretion takes place mainly from the acinar and ductal cells of the salivary glands (2). Whole saliva is not a homogeneous fluid and is made up of secretions from a number of sources, including fluid from the extrinsic glands and intrinsic glands, epithelial cell secretions, and the gingival crevicular fluid. In addition, capillaries passing through the salivary glands facilitate the entry of analytes from the systemic circulation into saliva (1).

THE SALIVARY GLANDS

The salivary gland systems in humans are divided into two separate exocrine groups: major and minor glands. Major salivary glands are bilateral paired glands and include parotid (PG), submandibular (SMG), and sublingual glands (SLG) [Figure 1]. There are numerous minor salivary glands as well. Salivary glands are associated with ducts and the gland is internally divided into lobules. Nerves and blood vessels enter the glands at the hilum and steadily branch out into the lobules. The lumina are formed by intercalated ducts in the duct system, and ending up in the secretory ducts. Human salivary glands terminate in the mouth. All salivary glands together produce around 90% of the total volume of saliva (3). Organogenesis of salivary glands, include multiple cell types, such as epithelial, myoepithelial, mesenchymal, neuronal, lymphatic, and endothelial cells. The interactions among above mentioned cell types occur during the initiation and development of the salivary epithelium (4).

Parotid glands (PG)

The largest salivary gland is PG and is found wrapped around the mandibular ramus. Mainly this paired gland secretes saliva of serous nature and enters the oral cavity via Stensen’s duct (4).

Submandibular glands (SMG)

The SMG is also a paired gland located beneath the lower jaw, superior to the digastric muscles. SMG secretion produces a mixture of serous and mucus fluid, entering the oral cavity via Wharton's ducts, and about 70% of saliva production in the oral cavity is produced by the SMG, although SMG is much smaller than the PG (4).
Sublingual gland (SLG)

The paired SLG is located beneath the tongue, anterior to the SMG, and contributes to 5% of saliva production in the mouth (4). The main secretion is mucus in nature. Unlike the PG and SMG, the ductal system of the SLG do not have striated ducts, and exit from 8-20 excretory ducts (4).

Minor salivary glands

In the oral cavity within the submucosa, there are over 600 minor salivary glands. The size of them is varies between 1–2mm in diameter and unlike the other glands. The minor glands are not encapsulated by connective tissues, only surrounded by it. Minor salivary glands have a common excretory duct together with another gland or they have their own excretory duct (4).

Von Ebner's glands

Von Ebner's glands are found in circumvallate papillae of the tongue. They secrete saliva of serous nature and facilitate the perception of taste (4).
INNERVATION ROUTES OF SALIVARY GLANDS

It has been known for the last 150 years that nervous to the salivary glands control the secretion of saliva, and both parasympathetic and sympathetic arms of the autonomic nervous system innervate the salivary glands resulting in increased amylase output and volume flow (6).

Parasympathetic innervation to the salivary glands: Innervation of the PG occurs via the cranial nerve IX: the glossopharyngeal nerve. The SMG and SLG obtain their parasympathetic input from the cranial nerve VII: facial nerve through the submandibular ganglion (3).

Sympathetic innervation to the salivary glands: The sympathetic salivary centers are located in the upper thoracic segments of the spinal cord and the sympathetic innervation of the salivary glands occurs through the thoracic segments T1-T3, leading to an increase in saliva secretion. Additionally, the sympathetic nervous system affects salivary gland secretions indirectly by innervating the blood vessels, which supply the glands (3).

The ultimate results, of both parasympathetic and sympathetic stimuli, are an increase in salivary gland secretion but it is the parasympathetic system that provides the main control of the salivary glands (3). The saliva coming from the parasympathetic system is watery, contains fewer proteins and has a high flow rate, whereas sympathetic regulation contributes saliva with higher viscosity due to mucin-rich saliva with a low flow rate (7).

STIMULATED VERSUS UNSTIMULATED WHOLE SALIVA

Unstimulated whole saliva, also called resting saliva, is composed mainly of SMG saliva together with saliva from SLG and minor salivary glands. The characteristics of unstimulated saliva are that it is more viscous and is mucin-rich (7). Normal unstimulated saliva secretion: > 0.25 ml/min

Hyposalivation: < 0.1 ml/min

Stimulated whole saliva is mainly composed of PG saliva and to some extent saliva from the SMG that is produced upon stimulation. Characteristics of stimulated saliva reveal that it is thin, watery and amylase-rich (7). Normal stimulated saliva secretion: > 1 ml/min

Hyposalivation: < 0.7 ml/min

MAIN FUNCTIONS OF SALIVA

Whole saliva has multiple functions, include rinsing, solubilization of food substances, food and bacterial clearance, lubrication of soft tissues, bolus formation, dilution of detritus, swallowing, speech, facilitation of mastication, and through a number of mechanisms saliva protects the teeth, all
of which are related to its fluid characteristics and specific components. In addition, saliva components contribute to mucosal coating, digestion and antibacterial defence (8).

**Digestion**

Saliva is essential for the normal mastication of food, for moistening food and helping to create a food bolus. The lubricating function of saliva allows the food bolus to be passed easily from the mouth into the esophagus. Food intake can be difficult and uncomfortable if there is a lack of salivary lubrication. Saliva contains important enzymes, such as salivary amylase involved in the first conversion of polysaccharide carbohydrates and breakdown of starch into simpler sugars that can later be absorbed or further broken down in the small intestine, and lingual lipase, which initiates the breakdown of lipids and has a protective function, helping to prevent bacterial build-up on the teeth and washing away adhered food particles (7, 9).

**Disinfectants**

It is well known that saliva contains natural disinfectants, such as locally and systemically produced immunoglobulins, lysozyme, lactoferrin, peroxidase, mucins, and a collection of antimicrobial peptides (10). Salivary immunoglobulins, in particular secretory immunoglobulin A (sIgA) is the dominant form in oral mucous secretions, unlike in systemic circulation where immunoglobulin G (IgG) is more abundant (10-11). Secretory IgA in the oral cavity acts as a first line of defense in the innate immune arsenal by immobilizing antigens, such as bacteria (12). IgG enters the oral cavity through the salivary glands, simply passing through the epithelium but during oral inflammatory conditions, such as periodontal disease, monomeric IgG enters saliva mainly via gingival crevicular fluid (GCF) and thus not through the salivary glands (13).

Antimicrobial peptides (AMPs), a family of antimicrobial agents, a major component of the innate defense mechanism, have been identified in most physiological fluids and in an exposed environment, such as the mouth, where they play an essential role in the balance between health and disease (14).

Peroxidase and lysozymes are common in different secretions, such as saliva and tears. They have strong antimicrobial activity, which cause damage to the cell walls of bacteria by hydrolysis (15).

Mucins are glycoproteins that are viscous and rather insoluble. The main role of mucins is to protect the soft tissues of the oral cavity from dehydration and mechanical damages. In addition, mucins are responsible for the selective adhesion of bacterial and fungal agents and help to prevent biofilm formation. They also protect both soft and hard tissues from abrasion damage by forming a lubricating coating on tissues (15).
PERIODONTAL DISEASE

The terminology of periodontal disease encompasses gingivitis, chronic periodontitis and aggressive periodontitis according to the International Workshop for the Classification of Periodontal Disease (1999). There are numerous subcategories as well: periodontitis as a manifestation of systemic disease, necrotizing periodontal disease, abscesses of the periodontium, periodontitis associated with endodontic lesions, and development of acquired deformities and conditions (16).

Gingivitis

Gingivitis is an inflammatory condition in the periodontal tissue caused by accumulation of dental plaque but it does not include any type of soft tissue- and bone loss surrounding the teeth. There are two forms of gingivitis: plaque induced gingivitis and non-plaque induced gingivitis. The most common type is plaque induced gingivitis with the presence of inflammation in the gum. Clinical features of induced gingivitis are redness, swelling, and bleeding, this condition is reversible and can usually be reversed by treatment and adequate oral care maintenance (16).

Chronic Periodontitis

Periodontitis is a chronic inflammatory disorder, associated with complex of interactions between periodontal bacteria, the host inflammatory response, genetic, environmental and behavioral risk factors. The most common form of the disease is plaque induced periodontitis, characterised by gingival inflammation, release of different pro-inflammatory cytokines, destruction of periodontal tissues and alveolar bone. Clinical signs of periodontitis are pocket formation, swollen and bleeding gingiva. Chronic periodontitis is a slowly progressing disease but it can include episodes of more rapid progress. The supporting collagen of the periodontium degenerates, resorbing alveolar bone, and the gingival epithelium migrates along the tooth surface and ultimately forming a periodontal lesion. Finally, the outcome of untreated periodontitis is tooth loss (17, 18) [Figure 2].

In this condition, predominantly anaerobic bacteria are the initiating agents, the red complex essentially: Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola, together with Aggregatibacter actinomycetemcomitans and some members of the orange complex, such as Prevotella intermedia (19). The process starts with bacterial adhesion and colonization of the teeth surface and accumulation of the dental biofilm. Periodontal pathogens have the ability to penetrate the gingival epithelium and release endotoxins and cytotoxic enzymes, and by-products that could induce breakdown of the extracellular matrix (ECM). Bacterial toxins activate several proteolytic enzymes and an inflammatory response. The damage caused by pathogens initiate directly or indirectly by trigging host-mediated responses that finally lead to tissue breakdown. In a periodontal lesion, neutrophilic granulocytes act first, followed by antibody activation of lymphocytes and monocytes (18).
Risk factors of periodontitis

Periodontitis is considered a complex interaction of bacterial infection, host response and other important factors. Common risk factors associated with periodontitis, include presence and amount of the periopathogens, smoking, genetics, obesity, osteoporosis, low dietary calcium and vitamin D, and poorly controlled diabetes (20).

![Figure 2: Model of pathogenesis of human periodontitis. PMNs: polymorphonuclear leukocytes, LPS: lipopolysaccharide, MMPs: matrix metalloproteinases (18) Reproduced by permission of John Wiley and Sons.]

Periodontal diagnosis

Diagnosis (Greek diágnōsis, examination/ analysis) is very important in terms of subsequent management of a patient’s disease / condition. Periodontal diagnosis is based upon clinical examination and radiographic assessments (16).

The clinical examination features of periodontitis include the presence of dental plaque, bleeding on probing, probing pocket depth, furcation involvement, clinical attachment loss, tooth mobility and presence of calculus.

The radiographic examination assesses the marginal bone level.

Chronic periodontitis is divided into localized and generalized forms depending on the percentage of sites affected based on the results of both clinical and radiographic examinations (16).

Localized periodontitis: up to 30% of the teeth are affected
Generalized periodontitis: >30% of the teeth are involved

Further, the severity of the disease is divided into three sub categories based to the amount of clinical attachment loss (16):
Slight: 1–2 mm
Moderate: 3–4 mm
Severe: ≥ 5 mm

16
CARDIOVASCULAR DISEASE

PREVALENCE OF CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD), including myocardial infarction (MI) is common in the adult population worldwide and a leading cause of mortality, accounting for half of all deaths. About 4 million deaths (47 % of all deaths) are causes by CVD in the European population each year and 1.9 million (40 %) within the European Union. In general, the mortality rate caused by CVD is higher in women (54 % of all deaths) than in men (43 % of all deaths). In Sweden, the percentage of deaths, which are from CVD follows: 41 % in women and 39 % in men (21). The incidence of acute MI (AMI) as well as the mortality rate after AMI has decreased over the past 20 years in Sweden (22, 23) [Figures 3 & 4]. This is explained by improved primary and secondary prevention, as well as of improved acute coronary care (22).

Figure 3: Age-standardized incidence of AMI per 100 000 individuals aged 20 years and above, by gender and year, 1988-2012. Blue line: men; Red line: women (22) Reproduced by permission of The National Board of Health and Welfare (Socialstyrelsen)
Myocardial infarction (MI)

An event of acute coronary syndromes (ACS) is mainly based on clinical- and electrocardiogram (ECG) findings and serological biomarkers, and is divided into three different entities; ST-elevation MI, non-ST-elevation MI or unstable angina. An AMI occurs when the coronary artery oxygen-rich blood flow to a part of the heart muscle suddenly becomes blocked leading to myocardial necrosis, with leakage of myocardial muscle proteins into the blood (24). According to the third universal definition, a MI is based on patient symptoms, specific biomarker elevation, ischemic ECG changes, and imaging evidence. Pathologically MI is defined as myocardial cell death. The clinical diagnosis criteria of MI include the following: a rise and/or fall of cardiac biomarker values, such as Troponin, symptoms of ischemia, detection of pathological Q waves in the ECG, finding of ST-segment changes, loss of viable myocardium or regional wall motion abnormality and angiographic identification of an intracoronary thrombus (25). There are five types of MIs and the classification is based on pathological, clinical, and prognostic differences (25) [Table 1].

<table>
<thead>
<tr>
<th>Table 1: The types of MIs</th>
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<tbody>
<tr>
<td><strong>Type 1:</strong> &amp; Spontaneous MI</td>
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<tr>
<td><strong>Type 2:</strong> &amp; MI secondary to an ischemic imbalance</td>
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<tr>
<td><strong>Type 3:</strong> &amp; MI resulting in death when biomarker values are unavailable</td>
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<tr>
<td><strong>Type 4a:</strong> &amp; MI related to percutaneous coronary intervention</td>
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<tr>
<td><strong>Type 4b:</strong> &amp; MI related to stent thrombosis</td>
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<tr>
<td><strong>Type 4c:</strong> &amp; MI related to restenosis</td>
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<tr>
<td><strong>Type 5:</strong> &amp; MI related to coronary artery bypass grafting</td>
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</table>
Atherosclerosis

Atherosclerosis is a disorder related to aging with a chronic inflammatory component. Inflammation is presumably involved in the initiation, progression and rupture of a coronary plaque ultimately leading to an infarction. An inflammatory reaction begins against endothelial cells and other components of the artery wall, and the inflammation sites attract accumulation of different cells, such as macrophages, T and B lymphocytes and mast cells [Figure 5]. Atherosclerosis is a multifactorial disease and involvement of different pathogens in the atherosclerosis process has also been suggested based on findings from epidemiological studies, such as *Chlamydia pneumonia*, *Helicobacter pylori*, periodontal pathogens (*Porphyromonas gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*) and *Cytomegalovirus* (26-29).

Figure 5: An illustration of development of atherosclerosis (30) Reproduced and modified by permission of Wolters Kluwer Health.

Risk factors of myocardial infarction

There are several known risk factor for MI. In fact it has been shown that nine factors can explain about 90% of all the risks for MI in several parts of the world. The INTERHEART study, a case-control study of MI, conducted in 52 countries, comprising a total number of 29 972 subjects (15 152 cases and 14 820 controls), revealed that about 90% of the risk associated with a myocardial infarction was explained by nine modifiable risk factors: smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, low consumption of fruit/vegetables and alcohol, level of physical activity, and enhanced ApoB/ApoA1 ratio (31).

Association between periodontal disease and cardiovascular disease

The association between periodontal disease and CVD has been discussed since late 80’s (32). A large number of epidemiological studies have shown an association between periodontitis and CVD (33), but a causal relationship has yet to be established (34). The link between periodontitis and CVD could be explained by periodontitis has effects to elevate systemic inflammation and endothelial dysfunction (33). Furthermore, pathogens derived from the oral biofilm involved in the pathogenesis of atheroma plaque formation (35). Previous studies showing improved oral hygiene and periodontal treatment may contribute to the prevention of atherosclerosis (36).
BIOMARKERS IN GENERAL

There are different definitions of a biomarker in the literature. The National Institutes of Health (NIH) defined a biomarker as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention” (37). According to the World Health Organization (WHO), the definition of a biomarker is following "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (38). Biomarkers are usable for diagnostics, monitoring disease progression, and disease monitoring and prediction. The term ‘biomarker’ is relatively new but it has have been used in preclinical research and clinical diagnosis for some considerable time (39). Examples of biomarkers include pulse and blood pressure through basic assessment, and timing among stimuli.

The functional outcome of IL-6 biomarker, for several clinical conditions has been observed. Amongst the expression is following: phase reactions, such as, monocytes, macrophages, fibroblasts, and dendritic cells. The functional outcome of IL-1β depends upon variables, such as concentration, duration of expression, and timing among stimuli. IL-6 has both pro- and anti-inflammatory properties, and is secreted by neutrophils, keratinocytes, fibroblasts and endothelial cells. The pro-inflammatory properties of IL-6 are to induce the acute-phase responses during inflammation. IL-6 appears to be the main mediator in various inflammatory diseases, such as renal disease and rheumatoid arthritis. As an anti-inflammatory cytokine, IL-6 inhibits the effects of TNF-α and IL-1. IL-8 is a member of the chemokine family, and produced by macrophages, epithelial cells and fibroblasts upon inflammatory stimulation. Primary function of IL-8 is to recruit neutrophils to the site of inflammation to phagocytose the antigen. Amongst all chemokines, IL-8 has significant potential as prognostic and / or predictive cancer biomarker, for several clinical conditions, as well as, for inflammatory conditions. TNF-α is a pro-inflammatory cytokine addition to being involved in local and systemic inflammation. It is a member of the group of cytokines that stimulate the acute phase reaction. It is produced by macrophages, although it can be produced by other cell types like lymphoid cells, mast cells, endothelial cells, and fibroblasts. The primary role of TNF-α is in the

More specifically, biomarkers are important mediators of inflammation, and they are associated with pathogenesis of many inflammatory diseases. Biomarkers are in clinical use, as non-invasive diagnostic markers, for disease prognosis and to monitor treatment response.

Inflammatory biomarkers

Many inflammatory biomarkers associated with oral diseases have been analyzed in saliva, such as, interleukin (IL) -1β, IL-6 and IL-8, tumour necrosis factor-alpha (TNF-α), matrix metalloproteinases (MMP) -8 and -9, TIMP-1, and myeloperoxidase (MPO) (1, 41, 42). Inflammatory mediators are released from different cells due to inflammatory conditions.

IL-1β is a pro-inflammatory cytokine that participates in immune defense against infection. The IL-1 cytokine family comprises eleven members, amongst which IL-1α and IL-1β are most studied. Both IL-1α and β have a 25% of sequence similarity at the protein level, despite being products of different genes. These proteins are produced by several types of cells, such as monocytes, macrophages, fibroblasts, and dendritic cells. The functional outcome of IL-1β depends upon variables, such as concentration, duration of expression, and timing among stimuli. IL-6 has both pro- and anti-inflammatory properties, and is secreted by neutrophils, keratinocytes, fibroblasts and endothelial cells. The pro-inflammatory properties of IL-6 are to induce the acute-phase responses during inflammation. IL-6 appears to be the main mediator in various inflammatory diseases, such as renal disease and rheumatoid arthritis. As an anti-inflammatory cytokine, IL-6 inhibits the effects of TNF-α and IL-1. IL-8 is a member of the chemokine family, and produced by macrophages, epithelial cells and fibroblasts upon inflammatory stimulation. Primary function of IL-8 is to recruit neutrophils to the site of inflammation to phagocytose the antigen. Amongst all chemokines, IL-8 has significant potential as prognostic and / or predictive cancer biomarker, for several clinical conditions, as well as, for inflammatory conditions. TNF-α is a pro-inflammatory cytokine addition to being involved in local and systemic inflammation. It is a member of the group of cytokines that stimulate the acute phase reaction. It is produced by macrophages, although it can be produced by other cell types like lymphoid cells, mast cells, endothelial cells, and fibroblasts. The primary role of TNF-α is in the
regulation of immune cells, it has capacity to induce fever, to induce apoptotic cell death, to induce sepsis, and induce inflammation (43-45).

MMPs are enzymes that have the ability to degrade almost all ECM proteins. Typically, they are secreted by different cell types, such as fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes (46). MMPs have different roles depending upon the condition. In normal physiological processes, MMPs take part in tissue development, remodelling and wound healing (47). In addition, MMPs, by processing various non-matrix bioactive substrates, have an anti-inflammatory (defensive) role and act on pathogenic (tissue destructive) of large number of different tissue destructive diseases and conditions (48).

There are 28 members of MMPs. 23 are found in human beings, and they are genetically distinct but structurally related. All MMPs are classified into six different subfamilies and the most efficient MMPs are collagenases since these cleave not only collagens I, II, and III, also other ECM molecules and various proteins. The second group is gelatinases and the main substrate of this subfamily is gelatin. Gelatinases cleave ECM molecules and basement membrane proteins (49, 50). The activation of MMPs can take place in different ways and there are several mechanisms that control MMP activity: enzyme inhibition, inflammatory cytokines, growth factors and hormones (51).

There are two major types of endogenous MMP inhibitors: Tissue inhibitors of matrix metalloproteinases (TIMPs) and $\alpha_2$– macroglobulins. The four different types of TIMPs are known: TIMP-1, -2, -3, and -4. The molecular structure of TIMPs, divided into two subdomains: C- and N-terminals, dictate the function properties of the molecule. The N- terminal domain directs the pathological activities of MMPs, by blocking the Zn$^{2+}$ reaching the active site of MMP molecule. The extent of inhibition differs between different MMP molecules (51). The balance between TIMPs and MMPs is challenging to maintain in normal physiological conditions in tissues (52).

MMP-8, also known as collagenase-2 or neutrophil collagenase, is particularly related to inflammatory conditions. MMP-8 is expressed mainly by neutrophils but also by several other cells, such as macrophages, plasma cells, T-cells, fibroblasts, chondrocytes and epithelial cells. It is stored in intracellular granules, and secreted and released in response to extracellular stimuli (53). MMP-8 is secreted as an inactive pro-enzyme and activation can be initiated by reactive trypsin or other MMPs (54, 55). TIMP-1 is the major inhibitor of MMP-8. Elevated MMP-8 concentrations are found in inflammatory conditions, such as periodontontal disease, dental caries, atherosclerosis, and cancer (56-58).

MPO is an enzyme released from PMN granules, is strongly associated with on-going inflammation (59), and has widely been used as an inflammatory marker of both acute and chronic conditions (60,61). The important role of MPO is to inactivate TIMPs and pathogenic bacteria by generating reactive oxygen species, as well as oxidatively activating latent proMMP-8 and -9 (59, 62).
Biomarkers of periodontal disease

Periodontal disease diagnosis is based upon clinical examination and radiographical assessments of periodontal tissues but tools for diagnosis, evaluation of severity and prognosis of periodontal disease are currently insufficient. Based on this statement, sensitive and disease-specific salivary biomarkers to complement regular clinical examination are of interest. A number of inflammatory biomarkers (IL-1β, IL-6, and IL-8, MMP-8, TIMP-1 and TNF-α) associated with oral diseases, such as dental caries, gingivitis and periodontitis have been detected in saliva. Whole saliva contains local- and systemic derivative biomarkers, which raises the possibility of using saliva as a diagnostic medium for periodontal disease. (41, 42, 63, 64). In addition, the use of disease-specific biomarkers increases the specificity and sensitivity for obtaining accurate diagnostic and prognostic information. Elevated salivary levels of IL-1β and MMP-8 are significantly associated with severe periodontitis compared to healthy controls (41) [Figure 6].

![Figure 6: Salivary diagnosis: Periodontitis and Cardiovascular Disease (65)
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Biomarkers of cardiovascular disease

In 1956, the first report on the use of cardiac biomarkers for MI was published, measuring aspartate aminotransferase (66). Since then improved diagnostic methods have been developed and since the year 2000 troponins are used to detect and define a MI (67). Biomarkers released from a myocardial injury of myocardial ischemia and necrosis, include cardiac troponins I (TnI) and T (TnT), creatine kinase-MB (CK-MB), total creatine kinase, myoglobin (MYO), N-terminal prohormone of brain natriuretic peptide (NT-pro BNP) and lactate dehydrogenase (68) [Figure 6]. Complimentary markers, such as Cystatin C and growth differentiation factor-15 (GDF-15) provide additional information for diagnosis, evaluation, risk assessment and prognosis of AMI patients (69-71).
Cystatin C levels are used as a clinical test for kidney function, produced by all nucleated cells and released to the blood (72). Increased levels of cystatin C are reported to be a strong predictor of CVD in chronic kidney disease patients (71). Yamashita et al reported a significant correlation between cystatin C levels and cardio-ankle vascular index (a marker of early-stage arteriosclerosis) in female subjects without chronic kidney disease (73).

GDF-15 is a novel emerging cardiovascular risk marker. It is well known that in healthy conditions, GDF-15 is expressed at low levels in all organs except the placenta but when it comes to pathological conditions related to inflammation, tissue injury and remodeling, the expression of GDF-15 increases rapidly. The main role of GDF-15 is to regulate inflammatory pathways in injured tissues (74). Higher levels of GDF-15 have been found in patients with chronic heart failure (70, 75).

NT-proBNP is synthesized and secreted from the cells of ventricular myocardium in response to pressure and volume overload of the heart. NT-proBNP is a peptide and acts as a hormone, which dilates the blood vessels and increases diuresis. There are number of clinical settings in which NT-proBNP levels are elevated, including acute and chronic heart failure, renal dysfunction and pulmonary disease, as well as, acute coronary syndrome (76). NT-proBNP is linked to prognosis and is a predictor of short and long term mortality in patients with heart failure, of future cardiac events and survival after AMI (69, 77).

Tn I & Tn T have become the golden standard for diagnosis of AMI since cardiac troponins are highly tissue specific for the myocardium. Further, they act as regulatory proteins of the myocardium and are required for muscle contraction (68). Analyses of cardiac troponins are also very sensitive, thus allowing detection of small myocardial injuries (78).

The use of saliva for diagnosis of AMI has been a popular area of interest and could potentially provide faster screening and verdicts. Floriano et al. reported that the use of a saliva-based Nano-Biochip test together with ECG, may provide a convenient and prompt screening method for cardiac events in pre-hospital stages for AMI patients. (79). In the same study the authors reported that cardiac enzymes such as CK-MB, MYO, TnI & T and inflammatory markers (C-reactive proteins, TNF-α, MMP-9 and MPO) in saliva were elevated in AMI patients (79).
SALIVA VERSUS BLOOD

Blood: serum or plasma is traditionally and most frequently the source of measurable biomarkers. Saliva is a multifaceted oral fluid, like blood it contains numerous components, including enzymes, hormones, antibodies, cytokines, and growth factors. Blood sampling and analysis can often be expensive, problematic and physically invasive. Using saliva as a medium for biomarker development and assessment eases patient discomfort by convenience and a non-invasive method of diagnostic purpose for diseases/conditions.

The advantages of saliva for the support of diagnostic potential include the following:

- Non-invasive procedure: painless and reduced discomfort
- Undemanding collection: saliva sampling can be done by anyone, including self-collection.
- Cost effective procedure
- Saliva is safer to handle, easier to store and ship than blood

They are several favorable attributes of using saliva as a diagnostic fluid, thus saliva offers some distinct advantages in selected situations. Some of the mediators detected in serum or plasma are also found in saliva but the levels of these mediators are substantially diminished (80, 81).
OBJECTIVES

The overall aim of this thesis was to explore and expand our knowledge on the utility of salivary biomarkers for detection of oral- and systemic diseases.

The specific aims of this thesis were:

- To explore salivary biomarkers for periodontal disease as a complement to clinical examinations in epidemiological surveys. (Study I)

- To explore salivary biomarkers for systemic diseases, to complement clinical examinations in epidemiological surveys. (Study II)

- To investigate whether salivary concentrations of selected cardiovascular biomarkers could be used to identify patients with a previous MI and if such markers in plasma and saliva were related to periodontal status. (Study III)

- To explore the levels of the inflammatory markers; MMP-8, -9, TIMP-1 and MPO in saliva, and their relationship to previous MI and periodontal disease. (Study IV)
MATERIALS AND METHODS

This thesis utilizes of two different projects: Skåne cohort and PAROKRANK sub-cohort.

SKÅNE COHORT – STUDY POPULATION (STUDIES I & II)

A randomly selected sample of 1000 adults living in Skåne, a county in the southern part of Sweden, was invited to participate in a clinical study of oral health. The individuals were 20–89 years old and were registered living in Skåne during 2007. The participant list was obtained from The Swedish Government’s Person Address Register and included background variables, such as gender, domicile, address and age in 5-year-intervals. Of the original sample, 11 individuals had moved from the region, 14 had an unknown address and nine were deceased, thus leaving 966 individuals as the initial sample. In total, 451 individuals were examined clinically 232 (51%) women and 219 (49%) men. The clinical examinations were performed between March 2007 and November 2008 and took place at the Faculty of Odontology at Malmö University, Sweden, and at three clinics of the Public Dental Service in Helsingborg, Kristianstad and Ystad, in Skåne. Dentists were coordinated regarding diagnostic criteria through comprehensive written instructions, practice and through discussing clinical cases.

Questionnaire

All the clinically examined participants were asked to respond to a questionnaire, and only one failed to answer. This consisted of 58 questions concerning patient perception of oral health, oral healthcare needs, pain, use of oral healthcare, dental materials and background factors (82). Further, anamnestic data including diseases, use of medication, smoking and snuffing habits were collected. Patients who were not able to or were not interested in being clinically examined were contacted by the phone to be part of a non-response analysis, and 175 of these individuals answered the same questionnaire as the individuals participating in the clinical study. Individuals who filled in the questionnaire as a non-response analysis were more likely to be born in Sweden, have a lower educational level and were missing a higher number of teeth than those participating in the clinical study. These differences can most likely be explained by the fact that the non-response group included most of the oldest individuals compared with the clinical study. The individuals, who neither participated in the clinical examination nor answered the questionnaire, were those who could not be reached by the phone or letter, were unable to take part due to bad health, due to old age or were simply not interested in participating in this study.

Clinical examination

The clinical examinations were performed by dentists, and 90.5% of the examinations were performed by four of those dentists, all employed at the Department of Oral Diagnostics, Faculty of Odontology, Malmö University. All patients were examined using typical examination procedures in standard surgeries. All clinical periodontal measurements were recorded at four sites (buccal mesial,
lingual and distal) on each tooth. Presence of visible plaque was recorded after drying with air. Probing pocket depth (PPD) was measured parallel to the tooth with a periodontal probe with 1mm grading (Hu-Friedy PCPUNC157). The deepest pocket ≥ 4 mm was registered. Bleeding on probing (BOP) was recorded after probing of the pockets. Third molars, root remnants that could not be used for restorations and dental implants were excluded in all measurements.

Radiographical examination

Digital panoramic and four bitewing radiographs taken on all participants were subjected to thorough examination. As a result, eight individuals were excluded: two subjects were edentulous, one person was edentulous but with dental implants, radiographs were missing from four individuals, and finally for one individual the radiographs were of an unacceptable quality. Therefore, the final study sample comprised of 443 participants (45.8% of the initial sample). To calculate intra-observer agreement, the observer performed a new assessment of the marginal bone level in 100 individuals. The radiographs were randomly chosen by another person to represent an even distribution of patients who had been classified in one of the three different categories.

The periodontal diagnosis was based on radiographic assessment only. Groups were defined as following:

PD– = loss of supporting bone tissue < 1/3 of the root length
PD = loss of supporting bone tissue ≥ 1/3 of the root length in < 30% of the teeth
PD+ = loss of supporting bone tissue ≥ 1/3 the root length in ≥ 30% of the teeth

Subjects with no or minor bone loss, i.e. PD- constituted 69% of the population. Twenty percent of the study population had marginal bone loss corresponding to localized periodontal disease (PD) and 11% exhibited generalized periodontal bone loss (PD+).

Saliva sampling and sample preparation for analysis

Sample collection: Stimulated saliva was collected over 5 minutes with chewing on 0.5g of paraffin into a graded test-tube. The collected amount was determined, excluding the foam. Saliva sampling and scoring of results was performed by trained dental assistants. Collected samples were immediately frozen at −20°C until processing. The present study includes 441 saliva samples from subjects with and without experience of periodontitis.

Sample preparation: The samples were thawed and centrifuged at 500 g for 10 minutes at 4°C. The supernatants were collected and divided into several aliquots that were quick frozen and stored at −20°C until analysis. Each aliquot was used only once in an assay and then discarded. The pellets, containing whole cells, were resuspended in 200μL PBS and transferred to 1.5 mL Eppendorf tubes (Eppendorf, Hauppauge, NY, USA), immediately frozen and stored at −80°C.
PAROKRANK is a multicentre, prospective, case-control study that was initiated and conducted by a group of researchers from the Departments of Medicine and Odontology at the Karolinska Institutet, Stockholm. 805 patients with MI and 805 randomly selected and matched controls were included. After initial examinations the participants were followed for two years [Figure 7].

**Figure 7: A schematic presentation of the PAROKRANK original study plan**

### Patients

In a sub cohort, saliva-based studies of PAROKRANK, a total number of 200 patients with previous AMI and 200 healthy controls (non-AMI) were recruited. Patients were consecutively enrolled between May 2010 and December 2011 from coronary care units at 11 hospitals around Sweden. Inclusion criteria included below 75 years with a first MI. Patients were introduced to the National Quality Registry for Suspect Acute Coronary Syndromes (RIKS-HIA). The registry for Secondary Prevention Following Coronary Intensive Care (SEPHIA), was used for follow-up eight to ten weeks after the index event. In addition, the data were collected via a web based case report form (CRF) developed specifically for this purpose. Selected data from this database was used in the present subgroup analyses.
Controls

Control subjects were randomly collected from the National Population Registry and were matched for age, gender and postal code. Exclusion criteria consisted of previous MI, cardiac valvular affected heart disease, and language barriers. Contact was established via phone by a study nurse at the coordinating centre (Karolinska University Hospital) providing information on the PAROKRANK project. Consenting persons underwent a structured interview on general health and were referred to a local centre for an extensive health and dental examination within one to two weeks of recruitment of the respective MI patient according to the principles outlined above for patients.

Examinations

Follow-up visit at Cardiology and Dental Departments

All patients and controls attended the local cardiology departments for laboratory measurements and questionnaire evaluation, and to the local dental department for extensive periodontal evaluation and salivary collection. Patients were examined eight to ten weeks after the MI and matched controls were examined within one to two weeks after the patients. All participants fasted and refrained from smoking for 12 hours prior to sampling. Morning medications were taken as usual with a small glass of water. Venous blood samples were collected in EDTA-coated tubes. The investigators performing the dental examinations were coordinated regarding examination procedure and diagnostic criteria through comprehensive written instructions.

Questionnaire

All participants in this investigation were asked to respond to a validated questionnaire, comprising of various questions related to family history, previous diseases in particular inflammatory disorders, diet, weight/waist circumference, glucometabolic perturbations, smoking, pulmonary diseases, socioeconomic factors, stress at work and home, depression and exhaustive syndromes, and information about general oral health status.

Dental examination

A standardised dental examination, including panorama x-ray was performed at hospital discharge of week’s eight to ten for the MI patients. The controls were investigated at the same local center during the same period, within one to two weeks after the matched patients.

Participants were examined clinically for number of present teeth, soft tissue pathologies, dental caries and periodontal status, including probing pocket depth of four sites per tooth, bleeding on probing and plaque score as present or absent, mobility and furcation involvement were recorded. The periodontal status of all participants was classified according to clinical criteria, such as number of pathological pockets ≥ 4 mm and gingival inflammation.
No data based on radiographic evaluations relating to alveolar bone level was available at the time of present subgroup analyses.

*Clinical periodontal diagnosis was based on:*

Gingivitis: bleeding on probing without loss of connective tissue attachment
Localized periodontitis: ≤ 30% of the teeth are affected
Generalized periodontitis: >30% of the teeth are involved
The severity of the disease was divided into three sub categories based to the amount of clinical attachment loss: Slight: 1–2 mm; Moderate: 3–4 mm; Severe: ≥ 5 mm

**Assessment of AMI**

The AMI was diagnosed by the physician in charge according to international definition of MI, (83,84) including the detection of a rise and/or fall of cardiac biomarkers, with at least one of the values being elevated (> 99th percentile upper reference limit), together with either typical symptoms/ ECG changes/ myocardial loss on imaging methods or thrombus on coronary angiography.

**Saliva sampling and sample preparation for analysis**

Sample collection: Stimulated saliva samples were obtained from all individuals in the sub cohort (200 cases and 200 controls) by chewing paraffin wax up to 10 minutes. The produced saliva was collected into a graded test-tube. The saliva collection continued until 2 mL of saliva was obtained or until 10 minutes had passed and the volume provided recorded if 2 mL was not obtained. The collected amount was determined, excluding the foam. Collected samples were immediately frozen at –20°C until processing.

Sample preparation: After completing the saliva sampling, each vial of saliva was centrifuged at 500g for 5 minutes, at 5°C, the supernatants were aliquoted into 1.5mL Eppendorf tubes (Eppendorf, Hauppauge, NY, USA), and stored at –80°C. Each saliva aliquot was used twice for the determination of selected biomarkers.

**Plasma samples**

Collected plasma samples were immediately frozen locally at –80 °C until transport to the central Bio-bank at Karolinska Institutet. EDTA plasma was used for determination of GDF-15, NT-proBNP and cystatin C concentrations. Plasma analyses included 60 samples in total from the study subjects.
**BIOCHEMICAL ANALYSES**

We have used different methods to analyze the biomarkers in saliva (*Studies I, II, III & IV*) and plasma (*Study III*). All the biochemical assays are described below.

In *Studies I & II*, the analyzed biomarkers were detected in more than 99% of the samples, except for TNF-α where the concentration was below the detection level in more than 50% of the samples. In *Study III*, we opted to use a routine method of analysis of Tn I in blood but we were unable to detect Tn I levels in stimulated saliva samples.

**ARCHITECT i4000SR SYSTEM (Abbott) (Study III)**

The ARCHITECT i4000SR SYSTEM (Abbott) assay is a chemiluminescent microparticle immunoassay used as a routine method for the quantitative determination of cardiac Tn I in human biological fluids. This assay was used in *Study III* according to the instructions of the manufacturer to determine salivary levels of Tn I.

In *Study III*, 25 (cases only- a pilot work) samples of saliva were analysed to determine Tn I levels.

**Bradford assay (Study I)**

The Bradford protein assay is a spectroscopic analytical procedure used to measure the concentration of protein in a solution. This assay was used in *Study I* according to the instructions of the manufacturer to determine the total protein concentration of the analyzed salivary biomarkers.

In *Study I*, 441 samples of saliva were analysed to determine total protein concentration.

**Enzyme-linked immunosorbent assay (ELISA) (Studies I-IV)**

ELISA is a test that uses antibodies and color change to identify a substance. ELISA is a popular biochemistry assay that uses a solid-phase enzyme to detect the presence of a substance, usually an antigen, in a liquid or wet sample. TIMP-1 in *Studies I & II* was measured by ELISA kits (Amersham Biotrak, GE Healthcare, Buckinghamshire, UK) and in *Study IV*, MMP-9 and TIMP-1 levels were determined by ELISA kits (Immundiagnostik, Bensheim, Germany) according to the manufacturer’s instructions. Lysozyme levels in *Studies I &II*, and salivary and plasma levels of cystatin C and GDF-15 in *Study III* were analyzed by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions (85).

In *Studies I & II*, 441 samples of saliva were analyzed to determine MMP-9, TIMP-1 and lysozyme levels.
In *Study III*, 400 (200 cases & 200 controls) samples of saliva were analyzed to determine cystatin C and GDF-15 levels.
In Study III, 60 (30 cases & 30 controls) samples of plasma were analysed to determine cystatin C and GDF-15 levels.

In Study IV, 400 (200 cases & 200 controls) samples of saliva were analyzed to determine MMP-9 and TIMP-1 levels.

**Immulite 2000 XPi (Siemens, Diagnostic Product Corporation) (Study III)**

Immulite 2000 XPi a routine method used for the quantitative measurement of NT-proBNP in heparinized plasma, as an aid in the diagnosis of individuals suspected of having congestive heart failure. The test is further indicated for the risk stratification of patients with acute coronary syndrome and congestive heart failure. The Immunlite 2000 XPi method was used in Study III to analyze the concentrations of NT-proBNP in plasma and saliva according to the manufacturer’s instructions.

In Study III, 60 (30 cases & 30 controls) samples of saliva were analyzed to determine NT-proBNP levels.

In Study III, 60 (30 cases & 30 controls) samples of plasma were analyzed to determine NT-proBNP levels.

**Luminex assay (Studies I & II)**

Luminex is a multiplex assay that simultaneously measures multiple analytes in a single run/cycle in a single sample volume of the assay. This assay is used for detection and quantification of proteins and peptides, or nucleic acids in biological fluids. In Studies I & II, Luminex assay (BioRAD, Hercules, CA, USA) was used according to the manufacturer’s instructions to analyzed TNF-α, IL-1β, IL-6 and IL-8 concentrations in saliva.

In Studies I & II, 441 samples of saliva were analyzed to determine TNF-α, IL-1β, -6 and -8 levels.

**Time-resolved Immunofluorometric assay (IFMA) (Studies I, II & IV)**

Time-resolved immunofluorometric assay (IFMA) is based on labeling immunoreactants with fluorescent probes. In Studies I, II & IV, MMP-8 concentrations in saliva were determined by IFMA according to the manufacturer’s instructions. The monoclonal MMP-8–specific antibodies (8708 and 8706) were used as capture and tracer antibodies, respectively (86, 87). The tracer antibody was labeled using europium–chelate. The assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM CaCl$_2$, 50 µM ZnCl$_2$, 0.5 % bovine serum albumin, 0.05 % sodium azide, and 20 mg/L diethylenetriaminepentaacetic acid. Samples were diluted in assay buffer and incubated for one hour, followed by incubation for one hour with tracer antibody. Enhancement solution was added, and after five minutes fluorescence was measured using a fluorometer.
In Studies I & II, 441 samples of saliva were analyzed to determine MMP-8 levels.

In Study IV, 400 (200 cases & 200 controls) samples of saliva were analyzed to determine MMP-8 levels.

**Western Blot (Study IV)**

Western blot or immunoblot is used to detect specific proteins, where antibodies are used to detect proteins in the sample. In Study IV, the presence and molecular forms of MMP-8 in saliva samples were analyzed using Western blotting according to the manufacturer’s instructions (86, 88). Samples were boiled in non-reducing Laemmli’s buffer, proteins were separated by a gel-electrophoresis, the proteins were semi-dry transferred to nitrocellulose membranes and incubated in 5 % milk powder. The membranes were first incubated with specific primary antibodies at room temperature overnight followed by incubation with secondary antibodies conjugated to horseradish peroxidase. The proteins were detected by incubation with an enhanced chemiluminescence technique and quantified with a Bio-Rad Model GS-700 imaging Densitometer using the Analyst™ program with correction for background values (86, 88).

In Study IV, 400 (200 cases & 200 controls) samples of saliva were analyzed to determine MMP-8 levels.

**STATISTICAL ANALYSES**

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 18 or 22 (SPSS Inc., Chicago, IL). Differences were considered significant at a probability level of p < 0.05.

In Study I, parametric tests were applied to calculate the characteristics and clinical variables between the groups. One-Way ANOVA and General Linear Model were used to compare biomarkers between the groups of periodontal health. The influence of smoking on salivary biomarkers was analyzed using a One-Way ANOVA test. A Tukey HSD Post-Hoc test was performed to calculate multiple comparisons between and within the groups of participants with and without experience of periodontitis.

In Study II, demographics of the study population was calculated by parametric tests. The significances of the differences in biomarker concentration between smoker/non-smoker, men/women and between those suffering with or without the indicated disease were calculated with Independent samples t-test. These data are expressed as mean ± standard deviation (SD). Significance of the differences, after compensation for age group, gender and smoking, was performed using the General Linear Model. The correlation between age and biomarker concentration was calculated with Pearson Product Moment Correlation test.

In Study III, parametric tests were applied to calculate the characteristics and clinical variables between the groups. Independent samples t-test was used for comparing mean values of the analyzed
biomarkers to determine the association of cardiac markers and systemic conditions. These data are expressed as mean ± SD. The correlation between periodontal status and biomarkers were calculated using Pearson- and Spearman’s correlation tests. Values under the detection limit were not included into the statistical analysis.

In Study IV, parametric tests were applied to calculate the characteristics and clinical variables between the groups. Independent samples t-test and General Linear Model were used to compare biomarkers between the groups were used to compare mean values of the analyzed biomarkers. These data are expressed as mean ± SD. The correlations were calculated by using Pearson- and Spearman’s correlation tests.

ETHICAL CONSIDERATIONS

Both projects conformed to good clinical practice guidelines and followed the recommendations of the Helsinki Declaration. All participants gave their written informed consent to the study protocols. The statistical analyzes were performed so that all participants were unidentifiable. Local Ethical Review Boards approved the protocol of the Skåne cohort and PAROKRANK project respectively.
MAIN RESULTS

SKÅNE COHORT

Baseline characteristics in Skåne population (Studies I and II)

The characteristics of the study population are presented in Table 2. There was a significant difference between men and women in regard to mental disorders and periodontal parameters, such as plaque and gingival indices, and PPD 4–5mm. The mean age, plaque- and gingival indices, PPD 4–5mm and > 5mm, the percentage of smokers, patients reporting heart disease, previously undergoing heart surgery, hypertension and bowel disease were higher amongst the male subjects.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Female n = 230</th>
<th>Male n = 218</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean ± SD)</td>
<td>47.9 ± 17.1</td>
<td>49.3 ± 16.9</td>
<td>0.39</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>16.1</td>
<td>17.8</td>
<td>0.63</td>
</tr>
<tr>
<td>No. of remaining teeth (Mean ± SD)</td>
<td>25.7 ± 3.6</td>
<td>25.1 ± 5.1</td>
<td>0.14</td>
</tr>
<tr>
<td>Plaque Index (Mean ± SD)</td>
<td>17.7 ± 19.7</td>
<td>26.8 ± 23.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Gingival Index (Mean ± SD)</td>
<td>26.3 ± 19.8</td>
<td>30.9 ± 21.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Probing Pocket depth 4–5mm (Mean ± SD)</td>
<td>6.1 ± 7.8</td>
<td>7.8 ± 9.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Probing Pocket depth &gt;5mm (Mean ± SD)</td>
<td>0.6 ± 1.8</td>
<td>0.7 ± 2.4</td>
<td>0.55</td>
</tr>
<tr>
<td>Heart surgery (%)</td>
<td>1.3</td>
<td>3.7</td>
<td>0.11</td>
</tr>
<tr>
<td>Heart disease (%)</td>
<td>7.5</td>
<td>8.4</td>
<td>0.74</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>14.2</td>
<td>20.5</td>
<td>0.08</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>3.9</td>
<td>3.2</td>
<td>0.68</td>
</tr>
<tr>
<td>Bowel disease (%)</td>
<td>6.6</td>
<td>7.4</td>
<td>0.74</td>
</tr>
<tr>
<td>Muscle and joint diseases (%)</td>
<td>25.7</td>
<td>20.5</td>
<td>0.19</td>
</tr>
<tr>
<td>Tumor (%)</td>
<td>4.0</td>
<td>3.3</td>
<td>0.68</td>
</tr>
<tr>
<td>Mental disorders (%)</td>
<td>8.8</td>
<td>2.8</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Table 3 demonstrates the demographic data related to periodontally healthy and affected subjects of the study population (n = 441). There was a significant difference between the groups with regards to all variables, except smoking and gingival index. The mean plaque and gingival indices, PPD 4–5mm and > 5mm, a reduction in the number of teeth and percentage of smokers were higher in the severe periodontitis group (PD+) compared with the other two groups. Participants reporting with hypertension, bowel diseases, tumors, diabetes, muscle and joint diseases were higher amongst the PD–subjects.

<table>
<thead>
<tr>
<th>Periodontal diagnosis/Variable</th>
<th>PD- n = 303</th>
<th>PD n = 89</th>
<th>PD+ n = 49</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean ± SD)</td>
<td>42.6 ± 15.5</td>
<td>59.9 ± 11.4</td>
<td>64.4 ± 11.8</td>
<td>0.001</td>
</tr>
<tr>
<td>No. of remaining teeth (Mean ± SD)</td>
<td>26.4 ± 3.53</td>
<td>25.0 ± 3.27</td>
<td>22.0 ± 4.82</td>
<td>0.001</td>
</tr>
<tr>
<td>Plaque Index (Mean ± SD)</td>
<td>19.8 ± 18.6</td>
<td>23.1 ± 23.7</td>
<td>35.5 ± 31.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Gingival Index (Mean ± SD)</td>
<td>28.1 ± 19.7</td>
<td>27.8 ± 19.1</td>
<td>34.8 ± 27.2</td>
<td>0.41</td>
</tr>
<tr>
<td>Probing Pocket depth 4–5 mm (Mean ± SD)</td>
<td>5.23 ± 7.32</td>
<td>8.51 ± 8.26</td>
<td>16.9 ± 13.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Probing Pocket depth &gt;5 mm (Mean ± SD)</td>
<td>0.19 ± 0.59</td>
<td>0.58 ± 1.46</td>
<td>4.31 ± 5.54</td>
<td>0.001</td>
</tr>
<tr>
<td>Heart surgery (n/ %)</td>
<td>2/ 0.7</td>
<td>4/ 4.5</td>
<td>5/ 10.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Heart disease (n/ %)</td>
<td>11/ 3.6</td>
<td>12/ 13.5</td>
<td>12/ 24.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypertension (n/ %)</td>
<td>33/ 10.9</td>
<td>26/ 29.2</td>
<td>17/ 34.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes (n/ %)</td>
<td>6/ 2.0</td>
<td>5/ 5.6</td>
<td>5/ 10.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Bowel diseases (n/ %)</td>
<td>17/ 5.6</td>
<td>6/ 6.7</td>
<td>8/ 16.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Muscle and joint diseases (n/ %)</td>
<td>53/ 17.5</td>
<td>31/ 34.8</td>
<td>18/ 36.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Tumor (n/ %)</td>
<td>7/ 2.3</td>
<td>3/ 3.4</td>
<td>6/ 12.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>16.2</td>
<td>13.5</td>
<td>28.6</td>
<td>0.06</td>
</tr>
</tbody>
</table>

In addition, all the analyzed biomarkers: IL-1β, -6 and -8, MMP-8, TIMP-1, lysozyme, MMP-8/TIMP-1 ratio and the total protein concentration from the three different patients groups were observed. The mean concentrations of IL-1β, MMP-8 and MMP-8/TIMP-1 ratio showed significant differences between the groups, and significance level remained after adjustment for gender, age and smoking. Additionally, multiple comparisons between and within the groups of participants with and without periodontitis with regards to IL-1β, MMP-8 and MMP-8/TIMP-1 ratio was studied, and the Tukey HSD Post-Hoc test showed significant differences between the PD- group and the PD+ group with severe periodontal disease.

The influence of tobacco smoking on the analyzed salivary biomarkers and the concentration of total protein were also studied. The results revealed that when the subjects were compared according to their smoking habits, significantly lower levels of IL-8, MMP-8/TIMP-1 ratio, and the total protein concentration were found in smokers. Smokers compared with non-smokers showed slightly lower concentrations of salivary MMP-8 (p = 0.052).
The figure shows the percentage of three patient groups based on the periodontal diagnosis [Figure 8].

![Figure 8: Percentage of three patient groups based on the periodontal diagnosis](image)

The correlation and significant levels between the clinical variables and analyzed biomarkers is demonstrated in Table 4. The most pronounced correlation was found between IL-1β and the number of deep pockets (>5 mm). TIMP-1 showed a negative correlation with BOP and the number of deep pockets (>5 mm). A number of analyzed biomarkers demonstrated a weak but statically significant correlation with the clinical variables.

| Table 4: The correlation (R) and p-values between the clinical variables and analyzed biomarkers |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| **Biomarker/ Clinical parameter**             | **IL-1β**                                      | **IL-8**                                      | **MMP-8**                                     | **TIMP-1**                                    | **MMP-8/TIMP-1**                              | **Total protein**                             |
|                                               | **R** (p)                                      | **R** (p)                                      | **R** (p)                                     | **R** (p)                                     | **R** (p)                                     | **R** (p)                                     |
| Visual plaque index                           | 0.3 (0.001)                                    | 0.1 (0.01)                                    | 0.1 (0.01)                                    | -0.1 (0.05)                                    | 0.2 (0.001)                                   | 0.1 (0.01)                                    |
| Bleeding on probing index                     | 0.3 (0.001)                                    | 0.1 (0.01)                                    | 0.2 (0.001)                                    | -0.1 (0.01)                                    | 0.2 (0.001)                                   | 0.2 (0.01)                                    |
| Probing Pocket depth 4–5 mm                   | 0.3 (0.001)                                    | 0.1 (0.001)                                   | 0.2 (0.001)                                    | 0.0 (0.296)                                    | 0.3 (0.001)                                   | 0.2 (0.001)                                   |
| Probing Pocket depth >5 mm                    | 0.4 (0.001)                                    | 0.1 (0.01)                                    | 0.1 (0.01)                                    | -0.1 (0.01)                                    | 0.2 (0.001)                                   | 0.2 (0.001)                                   |

**Study II**

The influence of gender, smoking and age on the concentration of analyzed salivary IL-1β, -6, -8, lysozyme, MMP-8, TIMP-1 and total protein concentration presented in Table 5. Mean salivary levels were higher in male subjects compared to female subjects but only IL-1β and -8 reached a significant level. Non-smokers compared to smokers had significantly elevated levels of IL-8, MMP-8, ratio of MMP-8/TIMP-1 and total protein concentration. All the analyzed biomarkers, except IL-6 and TIMP-1, correlated significantly with variable age.
Table 5: The influence of gender, smoking, and age on analyzed salivary biomarkers and total protein concentration

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Gender</th>
<th>Smoking</th>
<th>Correlation with age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female: n = 226</td>
<td>Yes: n = 75</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Male: n = 215</td>
<td>No: n = 366</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>62.7 ± 117</td>
<td>52.2 ± 74.8</td>
<td>0.07</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>7.5 ± 8.9</td>
<td>7.1 ± 7.9</td>
<td>0.47</td>
</tr>
<tr>
<td>IL-8 (pg/mL)</td>
<td>412 ± 446</td>
<td>342 ± 351</td>
<td>0.03</td>
</tr>
<tr>
<td>Lysozyme (ng/mL)</td>
<td>362 ± 360</td>
<td>416 ± 486</td>
<td>0.65</td>
</tr>
<tr>
<td>MMP-8 (ng/mL)</td>
<td>287 ± 235</td>
<td>239 ± 233</td>
<td>0.05</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>253 ± 193</td>
<td>288 ± 242</td>
<td>0.24</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>0.58 ± 0.76</td>
<td>0.35 ± 0.34</td>
<td>0.02</td>
</tr>
<tr>
<td>Total protein</td>
<td>833 ± 448</td>
<td>730 ± 403</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 6: Illustrates the mean salivary concentrations related to different systemic diseases/conditions of the study population. Patients reporting hypertension presented with higher concentrations of MMP-8 and lysozyme, as well as an increased MMP-8/TIMP-1 ratio but these differences were lost after adjustment for age group, gender and smoking habits. Diabetes patients had elevated concentrations of MMP-8 and a higher MMP-8/TIMP-1 ratio. These differences were more pronounced after compensation for age group, gender and smoking habits. Muscle and joint diseases were associated with increased levels of IL-1β, MMP-8 and the ratio MMP-8/TIMP-1. Furthermore, patients who responded that they were or had been suffering from any type of tumor had more than twice the concentration of IL-8 compared to those who had not had tumors. This difference was significant and remained after adjustment for age group, gender and smoking habits. Bowel disease was associated with elevated concentrations of IL-8 and total protein concentration. The patients that had previously undergone heart surgery had higher concentrations of MMP-8, and this difference remained after compensation for age group, gender and smoking habits. Variable inflammation, which included a combination of systemic conditions, such as heart disease, hypertension, bowel diseases, and muscle and joint diseases, was associated with increased levels of IL-8, MMP-8, the ratio of MMP-8/TIMP-1, lysozyme and total protein concentration.
Table 6: Mean salivary concentrations related to systemic diseases/conditions

<table>
<thead>
<tr>
<th>Condition/ Biomarker</th>
<th>Diabetes Mean ± SD</th>
<th>Hypertension Mean ± SD</th>
<th>Muscle/joint diseases Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (pg/mL)</td>
<td>65.0 ± 80.6 75.0 ± 118</td>
<td>94.5 ± 166 71 ± 103</td>
<td>101 ± 154 66.7 ± 102</td>
</tr>
<tr>
<td></td>
<td>p1= 0.6 p2= 0.4</td>
<td>p1= 0.1 p2= 0.9</td>
<td>p1= 0.008 p2= 0.02</td>
</tr>
<tr>
<td>MMP-8 (ng/mL)</td>
<td>481 ± 402 287 ± 258</td>
<td>371 ± 386 278 ± 231</td>
<td>356 ± 331 275 ± 241</td>
</tr>
<tr>
<td></td>
<td>p1= 0.08 p2= 0.02</td>
<td>p1= 0.006 p2= 0.41</td>
<td>p1= 0.007 p2= 0.04</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>1.76 ± 2.45 0.52 ± 0.70</td>
<td>0.78 ± 1.16 0.51 ± 0.77</td>
<td>0.77 ± 1.40 0.50 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>p1= 0.06 p2= 0.001</td>
<td>p1= 0.01 p2= 0.8</td>
<td>p1= 0.005 p2= 0.05</td>
</tr>
<tr>
<td>Lysozyme (ng/mL)</td>
<td>338 ± 172 398 ± 433</td>
<td>307 ± 325 414 ± 442</td>
<td>337 ± 265 414 ± 463</td>
</tr>
<tr>
<td></td>
<td>p1= 0.2 p2= 0.7</td>
<td>p1= 0.05 p2= 0.6</td>
<td>p1= 0.1 p2= 0.9</td>
</tr>
<tr>
<td>T.P.C (ug/mL)</td>
<td>867 ± 235 820 ± 434</td>
<td>877 ± 482 810 ± 416</td>
<td>901 ± 491 798 ± 406</td>
</tr>
<tr>
<td></td>
<td>p1= 0.5 p2= 0.9</td>
<td>p1= 0.2 p2= 0.9</td>
<td>p1= 0.03 p2= 0.1</td>
</tr>
</tbody>
</table>

*P1 indicates significance of the differences after a bivariate comparison; *P2 indicates the significance after compensation for differences in gender, age group and smoking habits.*

*T.P.C = Total protein concentration*
PAROKRANK SUB-COHORT

Baseline characteristics in the sub-cohort of PAROKRANK participants (Studies III and IV). Clinical characteristics and background variables of the study subjects are presented in Table 7. The number of smokers was significantly higher in the non-MI group. In MI patients, the mean value of dental caries was significantly higher compared to the non-MI subjects. The number of cases reporting hypertension, diabetes, respiratory disease, joint disease, showed all clinical periodontal parameters, and moderate and severe periodontitis patients were higher in the MI group. In non-MI subjects, the number of patients with gingivitis, mild periodontitis, kidney disease, cancer and bowel disease were higher in comparison to MI patients but it did not reach a significant level.

<table>
<thead>
<tr>
<th>Table 7: Demography of the study population in Studies III and IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Condition/Variable</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Age (Mean ± SD)</td>
</tr>
<tr>
<td>Gender (M) (n)</td>
</tr>
<tr>
<td>Smoking</td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Ex-smoker</td>
</tr>
<tr>
<td>Snuffing</td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Ex-snuffer</td>
</tr>
<tr>
<td>Dental caries (Mean ± SD)</td>
</tr>
<tr>
<td>Dental plaque (Mean ± SD)</td>
</tr>
<tr>
<td>BOP (Mean ± SD)</td>
</tr>
<tr>
<td>PPD 4–5 mm (Mean ± SD)</td>
</tr>
<tr>
<td>PPD ≥ 6 mm (Mean ± SD)</td>
</tr>
<tr>
<td>Total PPD mm (Mean ± SD)</td>
</tr>
<tr>
<td>Clinical periodontal diagnosis</td>
</tr>
<tr>
<td>Healthy (n/ %)</td>
</tr>
<tr>
<td>Gingivitis (n/ %)</td>
</tr>
<tr>
<td>Mild periodontitis (n/ %)</td>
</tr>
<tr>
<td>Moderate periodontitis (n/ %)</td>
</tr>
<tr>
<td>Severe periodontitis (n/ %)</td>
</tr>
<tr>
<td>Hypertension (n/ %)</td>
</tr>
<tr>
<td>Diabetes (n/ %)</td>
</tr>
<tr>
<td>Respiratory disease (n/ %)</td>
</tr>
<tr>
<td>Joint disease (n/ %)</td>
</tr>
<tr>
<td>Kidney disease (n/ %)</td>
</tr>
<tr>
<td>Cancer (n/ %)</td>
</tr>
<tr>
<td>Oral Cancer (n/ %)</td>
</tr>
<tr>
<td>Bowel disease (n/ %)</td>
</tr>
</tbody>
</table>
Study III

Plasma and salivary cardiac related markers are presented in Table 8. Salivary levels of cystatin C were significantly lower in MI patients. Apart from cystatin C no other analyzed salivary biomarkers differed between the MI and control group. Correlation analyses were performed to investigate the correlation between plasma and salivary cardiac related markers but there was no correlation between analyzed markers in saliva and plasma. The levels of NT-proBNP and GDF-15 were significantly higher in plasma of MI patients than control subjects.

Table 8: Mean concentrations and the correlation of biomarkers in plasma and stimulated saliva

<table>
<thead>
<tr>
<th>Condition/ Biomarker</th>
<th>MI Mean ± SD (n)</th>
<th>non-MI Mean ± SD (n)</th>
<th>p</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cystatin C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (ng/L)</td>
<td>922 ± 178 (30)</td>
<td>912 ± 192 (30)</td>
<td>0.83</td>
<td>0.1</td>
</tr>
<tr>
<td>Saliva (ng/L)</td>
<td>694 ± 331 (200)</td>
<td>715 ± 366 (200)</td>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>NT-proBNP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (ng/L)</td>
<td>310 ± 471 (30)</td>
<td>29.4 ± 48.2 (30)</td>
<td>0.001</td>
<td>0.2</td>
</tr>
<tr>
<td>Saliva (ng/L)</td>
<td>11.3 ± 12.5 (30)</td>
<td>13.5 ± 13.6 (30)</td>
<td>0.48</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>GDF-15</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (ng/L)</td>
<td>983 ± 613 (30)</td>
<td>625 ± 280 (30)</td>
<td>0.005</td>
<td>0.2</td>
</tr>
<tr>
<td>Saliva (ng/L)</td>
<td>69.4 ± 84.9 (200)</td>
<td>76.2 ± 105 (200)</td>
<td>0.48</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 9 shows the correlation between periodontal status and the analyzed biomarkers in MI patients and non-MI subjects. A negative correlation was found in the presence of dental plaque and salivary cystatin C levels in MI patients. The variables: BOP, PPD 4–5 mm and ≥ 6 mm and the clinical periodontal diagnosis were significantly correlated with GDF-15 levels in saliva in both groups. In non-MI subjects, significant negative correlation with cystatin C levels in saliva was found in presence of plaque and BOP. Further, the clinical periodontal diagnosis correlated negatively with salivary NT-proBNP levels in the same group.

Table 9: The correlation between periodontal status and the salivary cardiac markers

<table>
<thead>
<tr>
<th>Salivary biomarker/ Clinical variable</th>
<th>Cystatin C (R/p) MI</th>
<th>non-MI</th>
<th>NT-proBNP (R/p) MI</th>
<th>non-MI</th>
<th>GDF-15 (R/p) MI</th>
<th>non-MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque</td>
<td>-0.2/0.02</td>
<td>-0.3/0.001</td>
<td>0.1/0.39</td>
<td>0.2/0.32</td>
<td>0.0/0.94</td>
<td>0.1/0.25</td>
</tr>
<tr>
<td>BOP</td>
<td>-0.1/0.46</td>
<td>-0.2/0.006</td>
<td>0.0/0.81</td>
<td>-0.2/0.34</td>
<td>0.1/0.01</td>
<td>0.2/0.09</td>
</tr>
<tr>
<td>PPD 4–5mm</td>
<td>0.0/0.89</td>
<td>-0.0/0.56</td>
<td>-0.1/0.74</td>
<td>-0.3/0.13</td>
<td>0.3/0.001</td>
<td>0.2/0.002</td>
</tr>
<tr>
<td>PPP ≥ 6mm</td>
<td>0.0/0.82</td>
<td>-0.1/0.33</td>
<td>-0.0/0.86</td>
<td>-0.2/0.29</td>
<td>0.2/0.01</td>
<td>0.2/0.02</td>
</tr>
<tr>
<td>Total amount of PPD (mm)</td>
<td>0.0/0.78</td>
<td>-0.1/0.16</td>
<td>-0.1/0.53</td>
<td>-0.3/0.13</td>
<td>0.1/0.09</td>
<td>0.2/0.007</td>
</tr>
<tr>
<td>C.P.D</td>
<td>0.1/0.14</td>
<td>0.0/0.60</td>
<td>0.0/0.93</td>
<td>-0.4/0.01</td>
<td>0.3/0.001</td>
<td>0.2/0.004</td>
</tr>
</tbody>
</table>

C.P.D = Clinical Periodontal diagnosis
The association of the analyzed salivary biomarkers and systemic conditions in MI and non-MI subjects are presented in Table 10. Significantly higher levels of salivary cystatin C was found in MI patients with hypertension than non-MI subjects with same condition. Mean salivary cystatin C, GDF-15 and NT-proBNP levels were higher in MI patients with respiratory disease as compared to the controls but only cystatin C and NT-proBNP reached a significant level. In addition, salivary NT-proBNP levels were higher in MI patients who felt stressed at work in comparison to non-MI patients, although not statistically significant. The levels of cystatin C, GDF-15 and NT-proBNP in plasma were significantly higher in MI patients with respiratory disease, reported hypertension and who felt stressed at work than in controls. MI patients reporting with respiratory disease had significantly elevated levels of cystatin C in plasma than in control subjects.

### Table 10: The association of the analyzed salivary biomarkers and systemic conditions in MI and non-MI subjects

<table>
<thead>
<tr>
<th>Biomarker/Condition</th>
<th>Saliva: Cystatin C Mean ± SD (n)</th>
<th>p</th>
<th>Saliva: NT-proBNP Mean ± SD (n)</th>
<th>p</th>
<th>Saliva: GDF-15 Mean ± SD (n)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes MI</td>
<td>784 ± 338 (75)</td>
<td>0.05</td>
<td>16 ± 12.8 (11)</td>
<td>0.72</td>
<td>69.1 ± 75.0 (75)</td>
<td>0.20</td>
</tr>
<tr>
<td>No MI</td>
<td>651 ± 300 (100)</td>
<td>0.21</td>
<td>15.6 ± 12.5 (14)</td>
<td>0.53</td>
<td>73.5 ± 96.1 (100)</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Respiratory disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes MI</td>
<td>813 ± 379 (32)</td>
<td>0.01</td>
<td>6.8 ± 11.8 (3)</td>
<td>0.04</td>
<td>72.0 ± 74.7 (32)</td>
<td>0.56</td>
</tr>
<tr>
<td>No MI</td>
<td>661 ± 314 (161)</td>
<td>0.13</td>
<td>14.7 ± 12.5 (25)</td>
<td>0.81</td>
<td>70.4 ± 88.1 (161)</td>
<td>0.44</td>
</tr>
<tr>
<td><strong>Stressed at work</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes MI</td>
<td>663 ± 327 (121)</td>
<td>0.91</td>
<td>15.9 ± 12.4 (17)</td>
<td>0.17</td>
<td>73.8 ± 89.5 (121)</td>
<td>0.58</td>
</tr>
<tr>
<td>No MI</td>
<td>734 ± 317 (77)</td>
<td>0.38</td>
<td>11.6 ± 12.2 (12)</td>
<td>0.09</td>
<td>63.3 ± 78.2 (77)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

**Study IV**

Mean salivary levels of MMP-8 and -9, MPO, TIMP-1 and the ratios of MMP-8 and -9/ TIMP-1 from MI and non-MI subjects is illustrated in Table 11. The results showed that salivary MMP-8, MPO and TIMP-1 levels were higher in non-MI subjects compared to patients with MI but only MMP-8 reached a significant level and the difference was remained after adjustment for smoking, BOP, PPD 4–5mm and ≥ 6mm. MPO levels were slightly significantly elevated in the non-MI group but the difference was clearer after compensation for smoking and periodontal parameters. MMP-9/ TIMP-1 ratio were enhanced in MI patients relative to non-MI subjects. In addition, the different enzyme forms (pro- and active) of MMP-8 were observed by Western blot analyzes. The results revealed that
MMP-8, obtained as densitometric units from Western blot scanned images, did not differ between the study groups but there was a tendency for the active form MMP-8 to be somewhat higher in the non-MI group.

**Table 11:** The mean levels of MMP-8, -9, MPO, TIMP-1 and the ratio of MMP-8 and -9/TIMP-1 in stimulated saliva from MI and non-MI subjects

<table>
<thead>
<tr>
<th>Condition/ Biomarker</th>
<th>MI Mean ± SD</th>
<th>non-MI Mean ± SD</th>
<th>p1</th>
<th>p2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-8 (ng/mL)</td>
<td>440 ± 377</td>
<td>543 ± 398</td>
<td>0.008</td>
<td>0.003</td>
</tr>
<tr>
<td>MMP-9 (ng/mL)</td>
<td>260 ± 257</td>
<td>264 ± 217</td>
<td>0.88</td>
<td>0.78</td>
</tr>
<tr>
<td>MPO (ng/mL)</td>
<td>1637 ± 1386</td>
<td>1899 ± 1447</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>208 ± 119</td>
<td>229 ± 129</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>1.63 ± 2.79</td>
<td>1.62 ± 2.48</td>
<td>0.99</td>
<td>0.60</td>
</tr>
<tr>
<td>MMP-9/TIMP-1</td>
<td>0.74 ± 1.45</td>
<td>0.56 ± 0.81</td>
<td>0.12</td>
<td>0.20</td>
</tr>
</tbody>
</table>

p1 indicates significance of the differences after a bivariate comparison
p2 indicates the significance after compensation for differences in smoking, BOP, PPD 4–5 mm and PPD ≥ 6 mm.

Table 12 illustrates correlations between periodontal status, gender and smoking with analyzed biomarkers and the ratios. The results showed a weak but significant positive correlation between most of the analyzed biomarkers and the registered clinical variables in both study groups. There was a negative correlation between TIMP-1 and clinical variables. Further, some of the biomarkers showed weak correlation with smoking.

**Table 12:** The correlations of salivary MMP-8, -9, MPO, TIMP-1 and the ratios of MMP-8 and -9/TIMP-1 measurements, and periodontal parameters, gender and smoking in MI and non-MI subjects

<table>
<thead>
<tr>
<th>Clinical variable/ Biomarkers</th>
<th>MI</th>
<th>non-MI</th>
<th>PPD 4–5 mm</th>
<th>PPD ≥ 6 mm</th>
<th>C.P.D</th>
<th>Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP-8 (ng/mL)</strong></td>
<td>0.2**</td>
<td>0.4**</td>
<td>0.2*</td>
<td>0.2**</td>
<td>0.2**</td>
<td>0.2**</td>
</tr>
<tr>
<td><strong>MMP-9 (ng/mL)</strong></td>
<td>0.2**</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.2**</td>
<td>0.2**</td>
</tr>
<tr>
<td><strong>MPO (ng/mL)</strong></td>
<td>0.2**</td>
<td>0.3**</td>
<td>0.1</td>
<td>0.2*</td>
<td>0.2**</td>
<td>0.2**</td>
</tr>
<tr>
<td><strong>TIMP-1 (ng/mL)</strong></td>
<td>-0.2*</td>
<td>-0.2**</td>
<td>0.0</td>
<td>-0.1</td>
<td>-0.1</td>
<td>0.2*</td>
</tr>
<tr>
<td><strong>MMP-8/ TIMP-1</strong></td>
<td>0.3**</td>
<td>0.4**</td>
<td>0.2**</td>
<td>0.4**</td>
<td>0.3**</td>
<td>0.2*</td>
</tr>
<tr>
<td><strong>MMP-9/ TIMP-1</strong></td>
<td>0.3**</td>
<td>0.2**</td>
<td>0.1</td>
<td>0.4**</td>
<td>0.2**</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level**

C.P.D = Clinical Periodontal diagnosis
DISCUSSION

Salivary diagnostics is an emerging research field. To use saliva as a diagnostic medium for biomarker detection for local and systemic diseases is of high scientific interest both in dental and medical professionals. A literature search of PubMed using the following search terms: “saliva and biomarkers” presented nearly 2000 publications over the past 10 years, [Figure 9].

![Figure 9: Publications based on salivary diagnostics from 2003-2012](image)

Our findings related to the utilization of salivary biomarkers for the detection of oral and systemic diseases, in particular periodontal disease and MI. This may explore and improve current knowledge to provide a good platform for future studies.

Human saliva has many biological functions, offering some distinct advantages for monitoring and detection of a number of diseases, such as periodontal disease, dental caries, oral cancer, breast cancer, and human immunodeficiency virus (41, 42, 89-91). Several types of inflammatory biomarkers (IL-1β, -6 and -8, TNF-α, MMP-8 and -9, MPO) associated with both oral and systemic diseases have been detected in saliva using different analyzing methods (1, 63, 64, 92, 93). The composition of salivary proteins differs in stimulated and unstimulated whole saliva. Thus, it is suggested to use unstimulated saliva to analyze salivary proteins as the outcome affects the saliva collection method (94, 95).

In the study population of Skåne cohort, we investigated the possibility of using salivary biomarkers for oral (Study I) and systemic diseases (Study II) as a complement to clinical examinations. In Study I, IL-1β, MMP-8 and the ratio of MMP-8/TIMP-1 were found to be significantly enhanced in the saliva from subjects with severe periodontitis, suggesting that these components could be potential biomarkers for periodontitis/ local inflammation. Our findings regarding elevated salivary IL-1β and MMP-8 levels in periodontitis patients are in line with several previous studies (63, 96). IL-1β is an inflammatory biomarker and it remains to evaluate if it is possible to differentiate between gingivitis and periodontitis. Some studies have shown higher levels of IL-1β in GCF samples in sites with
periodontitis compared with GCF samples from gingivitis sites (97). One of the key biomarkers in inflammation process is MMP-8. Increased levels of MMP-8 in oral fluids are related to severity of periodontitis and it’s progression (98). Furthermore, chair-side tests measuring MMP-8 in mouth rinses have been shown to discriminate between gingivitis and periodontitis (99). Elevated levels of IL-1β and MMP-8 in GCF have also been associated with increased odds for subsequent attachment loss during periodontal maintenance (100). It is important to consider that analyses of GCF are site specific and saliva reflects the entire mouth. Furthermore, the salivary biomarkers analyzed in Study I were correlated significantly with most of the registered periodontal parameters. This could be an indication that the measured biomarkers reflect the degree of periodontal/gingival inflammation rather than periodontal tissue destruction. The findings in Study I support the proposal that saliva could be used in epidemiological surveys to detect oral inflammations.

Study II was performed to investigate selected salivary biomarkers (IL-1β, -6, -8, TNF-α, lysozyme, MMP-8 and TIMP-1) for detection of a few common systemic diseases. The results in this study showed that diabetes patients and patients who had undergone heart surgery had higher salivary MMP-8 levels. It is important to consider that patients who reported with previously undergone cardiac surgery and diabetes patients in this cohort were very few, eleven and sixteen respectively. Nevertheless, increased MMP-8 levels in association with diabetes have earlier been shown in saliva, GCF and in gingival tissues (98,101-104). The levels of IL-1β, IL-8 and MMP-8, as well as the MMP-8/TIMP-1 ratio were higher in subjects with muscle and joint diseases. Patients with bowel diseases had higher salivary levels of IL-8. Elevated salivary IL-8 levels in patients reported with experience of tumor diseases was found in our study, which is in line with a study performed by Wei and coauthors who investigated salivary IL-8 levels of patients with oral cancer (105). In addition, IL-8 has been shown to be elevated in both serum and saliva in patients with head and neck squamous cell cancer (106). Salivary IL-8 appears to be a good marker for evaluation of head and neck cancer, as well as in other clinical settings, such as muscle and joint diseases and inflammatory bowel disease.

In Study III, the cardiac related biomarkers (cystatin C, GDF-15, NT-proBNP and Tn I) were chosen for their involvement in CVD. The diagnostic utility in saliva to differentiate between patients having had an AMI eight to ten weeks earlier, and non-AMI subjects were explored. Our results showed that salivary cystatin C levels were significantly lower in MI patients but cystatin C levels in plasma were higher in patients with MI. It was challenging to analyze cystatin C in stimulated saliva samples as we found lower levels than expected. Timing of saliva collection, saliva collection method and different drugs taken by MI patients might have affected the salivary cystatin C levels. There are no previous studies relating cystatin C analysis in saliva of MI/CVD patients. Further studies are needed to investigate the diagnostic potential of salivary cystatin C in MI patients. In addition, we correlated cystatin C levels to periodontal status of the study population. The levels of cystatin C showed a weak negative correlation with dental plaque in both groups, with gingival inflammation in non-MI group. Earlier studies relating to salivary cystatin C in patients with periodontal disease revealed that gingivitis and periodontitis patients had higher levels of cystatin C in whole and parotid saliva compared to healthy controls (107,108), and periodontal treatment reduced the cystatin C levels in whole saliva (109). The explanation for these contradictory results could be that in our study population, they might had a lower severity of periodontitis.

Troponins in plasma are considered as the golden standard for diagnosis of MI. In our study (a pilot work, merely consist of 25 saliva samples of MI patients), we opted to use a same routine method for analysis of Tn I in blood for the analysis of saliva but we were unable to detect any Tn I levels in
stimulated saliva samples. This might be explained by the timing of the saliva sampling (eight to ten weeks after the onset of AMI) or due to the fact that Tn I might actually be undetectable in saliva. In general, Tn I levels reached their peak within 10 to 14 hours after an AMI. Increased troponin levels in plasma can be measured up to two weeks after an infarction. Previous investigations have revealed that salivary cardiac biomarkers can be used in the assessment of cardiac ischemia/necrosis (110,111).

Miller and co-authors analyzed Tn I in serum and unstimulated whole saliva collected from 92 AMI patients within 48 hours of chest pain onset and compared with healthy controls. The results from their study demonstrated that serum Tn I was the marker that was mostly associated with AMI. In saliva, Tn I levels were detectable in MI patients but it did not reach a significant level between AMI patients and controls (110). In another study, the authors used both stimulated and unstimulated whole saliva and serum from 30 AMI patients and 28 healthy individuals to detect Tn I levels (111). Saliva samples were obtained 12 and 24 hours after AMI. This investigation showed that in patients with AMI, serum and unstimulated saliva levels of Tn I, at both 12 and 24 hours of onset, were significantly higher than in controls. In addition, Tn I levels in AMI patients correlated significantly with serum and unstimulated saliva (111). In our study, we used an ELISA kit to analyze Tn I levels since Tn I levels were undetectable in the routine analyses but even with this method, we were unable to detect Tn I levels in saliva (data not presented; a pilot work, consisting of 20 saliva samples of MI patients). Further, in our study population, we experienced the same results when analyzing Tn T levels in stimulated saliva (data not reported). In both of the above-referred studies (110,111), they used unstimulated saliva samples for Tn I detection and saliva samples were collected within few hours after onset of AMI. Unfortunately, in these two studies, there were lacks of information regarding the oral status of the participants.

In our study, salivary NT-proBNP was measured using a highly sensitive routine method (Immulite 2000 XPi) the mean salivary NT-proBNP levels from MI patients were very low compared to plasma levels of NT-proBNP. In a previous study, salivary NT-proBNP was determined using AlphaLISA(R) immunoassay in patients with heart failure and healthy controls. The study was carried out to see whether NT-proBNP could be detected in saliva of patients with heart failure, and whether salivary levels correlated with plasma levels. The authors revealed that NT-proBNP could be detected in saliva of patients with heart failure but could not detect it in saliva samples from healthy controls. Furthermore, there was no correlation between salivary NT-proBNP and plasma NT-proBNP concentrations in heart failure patients (112) and this was in line with our results. In our study and the study performed by Foo et al. it is important to consider that the measurements of NT-proBNP were based on two different conditions, previous MI and heart failure respectively. However, in MI patients who reported being stressed at work had higher salivary levels of NT-proBNP.

Same pattern seen for NT-proBNP were also found for GDF-15 levels in saliva. The levels of GDF-15 were lower in saliva compared to plasma but in plasma GDF-15 levels were significantly elevated in MI patients compared to healthy controls. Despite of that, GDF-15 in saliva showed a weak but significant association with gingival and periodontal inflammation in both patients and controls. This demonstrates that periodontal health status has to be considered when analyzing GDF-15 in saliva samples and also in plasma.

Limited numbers of previous studies have demonstrated correlations between serum and salivary biomarkers of CVD (113,114). The medications used in the context of cardiovascular disease could be a possible explanation for the lack of correlation between salivary and plasma levels in our study. In
addition, the results could also have an affect due to saliva collection method together with timing of saliva sampling.

In Study IV, we examined following salivary biomarkers; MMP-8 and -9, MPO, TIMP-1 and the ratio of MMP-8 and -9/ TIMP-1 in previous MI patients and non-MI subjects with or without periodontal disease. MMP-8 is involved in periodontal disease progression and CVD. Earlier studies have reported that MMP-9 and MPO in plasma were associated with an increased risk, the onset and prognosis of CVD, particularly MI (115,116). In our study, salivary levels of MMP-8 and MPO were slightly higher in the non-MI group. This result differs to some extent from earlier studies. Furuholm et al. (117) showed that patients referred for open heart surgery had significantly higher salivary concentrations of MMP-8 compared to matched controls. No information about the reason for the open heart surgery or medication taken was given (117). A recent study, showed a lower concentration of MMP-8 in saliva, collected three to four days after the infarction from MI patients, which is in line with our findings (118). However, the same study also showed that the percentage of active MMP-8 was significantly higher in MI patients (118). This was not the case in our study, where we saw a tendency of more active MMP-8 in the non-MI group, despite using exactly the same techniques and anti MMP-8 antibody as Buduneli and co-authors. Further, the ratio of MMP-8 and -9/ TIMP-1 were marginally higher in MI patients but it did not reach a significant level. In another study, several inflammatory mediators in serum of patients with aortic sclerosis, related to severity of CVD were studied, and they detected elevated general MMP expression, and MMP-9 and TIMP-1 had a positive correlation with each other (119). In our study, MMP-9 correlated positively with MMP-8, MPO and the ratio of MMP-8 and -9/TIMP-1 but had a negative correlation with TIMP-1 levels. In previous animal studies, TIMP-1 has been shown to inhibit both the activity of MMP-9 during infarct healing and in the protection against infarct plaque rupture after AMI (120). MPO in plasma was associated with an increased risk of CVD (115). To our knowledge, there are no studies of salivary MPO in relation to MI, and our results showed that MPO in saliva strongly correlated with salivary MMP-8 and the ratio of MMP-8/ TIMP-1. According to previous reports, MPO exerted distinct protective role during inflammation, such as inactivation of pathogenic microbes and oxidative activation of latent proMMP-8 and -9 and inactivate TIMPs (60,121). Furthermore, we correlated the analyzed salivary biomarkers with periodontal status and smoking in both study groups. There was a weak but significantly positive correlation between most of the analyzed biomarkers and the registered clinical variables in both study groups. A negative correlation between TIMP-1 and clinical variables was noted but TIMP-1 correlated positively with smoking and it also reached a significant level in both groups. Furuholm and co-authors suggested that increased salivary levels of MMP-8 reflected periodontal disease activity in patients with coronary artery disease (CAD) compared to systemically healthy controls (117). Buduneli et al. and Mäntylä et al. suggested it may also reflect systemic inflammation, such as AMI (118,122). An earlier study demonstrated the GCF levels and associations between MPO and MMP-8 were related to development and treatment responses in patients with chronic periodontitis. The authors concluded that there was an interaction between the MPO oxidative pathway and MMP-8 activation, and this cascade might be useful as a potential biomarker for assessment of treatment outcomes (121).

One of the important risk factors of periodontitis is tobacco smoking. Our results from Study I showed that mean salivary IL-8 levels, MMP-8/TIMP-1 ratio and total protein concentration were significantly lower in smokers compared to non-smokers. Mean levels of TIMP-1 and lysozymes were enhanced in smokers. MMP-8/TIMP-1 ratio was low in smokers and a possible explanation could be that smoking down-regulated or reduced the expression and degranulation of MMP-8 by
non-neutrophil-lineage cells and neutrophils, respectively (123). The results in Study IV showed that smoking correlated positively with TIMP-1 levels both in MI and non-MI subjects. In addition, in the non-MI group, MMP-9 correlated significantly with smoking whilst the ratio MMP-9/TIMP-1 had a negative correlation with smoking. The impact of smoking on salivary biomarkers of periodontal disease may be explained by a direct effect on inflammatory cells and through their presence and activity, as well as a decreased flow of GCF in smokers leading to a decreased influx of inflammatory mediators into saliva (124).

Salivary biomarkers could be influenced by different medications. The discrepancy between the findings in our studies and previous studies could be due to the medication taken by our study populations. The subjects in Studies I and II with muscle and joint diseases were taking a number of analgesics, as well as immunosuppressive agents. Agents, such as non-steroidal anti-inflammatory drugs, paracetamol and opioids, are commonly used to treat chronic pain and inflammation could potentially interfere on the expression of certain biomarkers. Nevertheless, it is known that drugs like monoclonal anti-TNF medication and disease-modifying anti-rheumatic drugs exert various effects on salivary IL-1β, TNF-α and MMP-8 levels, including reducing and no effects respectively (125). The patients in Studies III and IV were taking a number different medications, such as statins, angiotensin-converting enzyme (ACE) inhibitors, beta blockers, anti-inflammatory drugs and thrombocyte inhibitory agents that could exert an effect on salivary cardiac and inflammatory biomarkers. According to previous studies, ACE inhibitors and statins potentially interfere on the expression of MPO, MMP-8 and -9. Some ACE inhibitors decrease the levels of MMP-9 both in acute and chronic phases of systemic conditions. Mechanisms of specific statins include reduction of MMP-9 levels (126,127).

A number of definitions of periodontal disease have been used in the literature over the years (16, 128-130), which was reflected in our study as well. In the Skåne cohort (Studies I and II), the study population was divided into three groups based on their periodontal disease experience. The groups were divided based on the amount of marginal bone loss around each remaining tooth and the periodontal diagnosis in this project was based entirely on radiographic assessment. The study population of PAROKRANK sub-cohort (Studies III and IV), periodontal diagnosis was based on clinical periodontal examination only. It is recommended to use both clinical examination and radiographic assessments for periodontal diagnosis. There is a higher risk to obtain false positive and negative results by using either clinical status or radiographic evaluation.

General aspects on strengths and limitations:
There are some important methodological considerations that have to be considered in relation to our findings. In Studies I & II, study design was cross-sectional, which comprised of cases and control individuals. Thus, there was potential selection and information bias, and it is recognized that the control individuals came from the same population base. Moreover, the medical status was retrospectively collected, which was based on the participants’ self-assessment and no verification of answers from anamnestic data. There could be a risk for false negatives, for instance undiagnosed diseases. In Studies III and IV, we collected data eight to ten weeks after exposure of an AMI and the post MI condition of the patients, including drugs used by the MI-group eventually exerted an effect on the analyzed biomarkers. There were many investigators (both dentists and dental hygienists), who performed dental examinations of the study population, which is a negative point as it could affect the outcome of clinical examinations. Another limitation is that periodontal classification of the study population in this sub-cohort of the PAROKRANK project was merely based on clinical periodontal
parameters and not together with radiographical assessment. Further, it was important to consider that the numbers of participants are limited in this sub-cohort.

In both cohorts, the results might have been affected by the pre-analytical procedures for saliva samples and the methods/ kits used for salivary analyses might not be highly sophisticated. However, there are both advantages and disadvantages to using biochemical methods for analyzing purposes. ARCHITECT i4000SR SYSTEM (Abbott) and Immulite 2000 XPi (Siemens, Diagnostic Product Corporation) are highly sensitive and gold standard routine methods for blood analyses of Tn I and NT-proBNP respectively. We have used both methods (ARCHITECT i4000SR SYSTEM: Tn I) and (Immulite 2000 XPi: NT-proBNP) for analysis in stimulated saliva samples. Antibodies used in both assays were very specific. These are automated methods, and systematically errors are obtained very rarely compared to other immunochemistry methods. Although both of the above mentioned methods are highly sophisticated, there could be a minimal risk to obtain analytical interference, which could distract antibodies in vitro immunoassays.

Bradford protein assay, ELISA, Luminex and IFMA assays, and Western blot are immunochemistry methods, are standardized and widely used in research laboratories. IFMA is a highly sensitive and sophisticated method compared to ELISA and Luminex methods. Luminex assays are a good choice for multiple analytes but these assays are less sensitive than ELISA assays. Western blot is a widely used analytical technique to detect specific proteins in a sample/ tissue/ extract. It is significant to consider that there are some limitations of using the above mentioned analytical techniques, even though reagents are formulated to minimize the risk of interference, the potential interactions with in vitro immunoassays and test components can occur, and well-trained laboratory assistants are needed to handle the samples to acquire good results.

Strength with the Skåne cohort is the large size, including 400 patients. Furthermore the periodontal diagnosis was based on radiographic assessments performed by few investigators. Major strength in PAROKRANK study is the carefully matching of cases and controls and cases were included at the occasion of a first MI, minimizing interactions of other compounding diseases and complications. The timing of the blood and saliva collections, eight to ten weeks after the MI, could in some context, be regarded as a strength, minimizing the acute stress response on inflammatory markers and other prognostically important biomarkers. Future studies, will address such circumstances.

**FUTURE IMPLICATIONS**

Salivary diagnostics is an evolving research area. Biomarkers found in saliva are mainly from plasma, serum, GCF and mucosal transudate, which infiltrate saliva due to oral and/systemic diseases. Highly sophisticated techniques are needed to provide the sensitivity and specificity for accurate and reproducible disease diagnosis. For example, to identify key molecules that appear in saliva during the processes of different conditions/ diseases is critical to the field of salivary diagnostics. The utility of saliva as a diagnostic tool will only be achieved once key biomarkers are validated in longitudinal studies.
Biomarkers related to inflammation, tissue destruction and bone remodeling associated with periodontitis appear in saliva but the evidence is insufficient in both stimulated- and unstimulated saliva. Clinical examination and radiographical assessments are standard procedures of diagnosis of periodontitis however there is a lack of investigations including patients with different periodontal disease phases. Future studies should aim to include patients with different phases of active disease to be able to identify more specific disease biomarkers in saliva. An easily fast taken saliva sample might be an attractive medium to traditional time consuming clinical diagnosis procedures of periodontal disease by offering some distinct advantages for screening, monitoring and detection.

Cardiac related biomarkers, in particular inflammatory markers, plaque adhesion/rupture and tissue necrosis are at insignificant levels in saliva during early hours after onset of an acute MI but the evidence is not robust and there is a lack of information regarding oral status in previous studies. AMI is a condition, which needs immediate therapy, salivary diagnostics might be perfect for a fast early detection but additional studies are required to evaluate salivary cardiac related markers measured in samples collected at the time of admission to identify an acute coronary event. Troponin levels in saliva were at insignificant levels eight to ten weeks after a MI. Before troponin levels are discouraged as a screening biomarker in saliva, further investigations are needed, including analyses of both stimulated and unstimulated saliva since troponin levels are measurable in saliva over the first hours of an AMI.

Certain salivary biomarkers, such as IL-1β, and -8, GDF-15, NT-proBNP and MMP-8 are highly measurable in whole saliva. Theses markers could therefore be highly interesting as screening markers in saliva, since all of them are related to conditions, such as cardiovascular disease and cancer. For instance elevated inflammatory markers as IL-8 correlate with an unspecific cancer diagnosis. Further studies are needed to develop key molecular biomarkers that correlated to a more specific cancer diagnosis.

Furthermore, type of saliva; stimulated or unstimulated will have a significant effect on salivary biomarker composition and analysis and this needs to be taken in to consideration when using saliva for diagnostic/screening purposes.

Future developments in salivary diagnostics for screening, risk assessment and therapeutic management for a range of conditions are promising and this approach will hopefully allow individualized treatments before arise of major clinical symptoms.
CONCLUSIONS

- Salivary IL-1β, MMP-8 and the ratio of MMP-8/TIMP-1 could be used as markers for periodontal disease in larger patient populations.

- Salivary IL-8 concentration was found to be remarkably high in subjects who had experienced tumor diseases and bowel disease. MMP-8 levels were elevated in stimulated saliva from patients after cardiac surgery or suffering from diabetes. The levels of IL-1β, IL-8 and MMP-8, as well as the MMP-8/TIMP-1 ratio were higher in subjects with muscle and joint diseases.

- Salivary GDF-15 levels correlated with periodontal status of MI-patients and controls, and the levels of cystatin C, NT-pro BNP and GDF-15 in saliva were higher in MI patients with other co-morbidities. The analyzed cardiac related biomarkers seem to be in very low concentrations in stimulated saliva and do not correlate to plasma levels.

- Tn I levels in stimulated saliva were undetectable.

- Salivary MMP-8 and -9, MPO, TIMP-1 and ratio of MMP-8, -9/ TIMP-1 correlated with each other and with periodontal parameters in both patients with or without previous MI.

- Saliva-based clinical testing could provide a future potential diagnostic tool for the detection of certain diseases/ conditions using biomarkers associated with enhanced oral and systemic inflammation but when evaluating biomarkers in saliva the influence of both systemic and oral health should be considered.
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