UNIVERSITY OF TURKU

SALIVARY DIAGNOSTICS IN PERIODONTOLOGY:
Salivary biomarkers in relation to periodontal status

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ABSTRACT

Periodontitis is an infection-driven inflammatory disease affecting tooth-supporting tissues, i.e., the periodontium. Whole saliva, an easily obtained specimen, is attractive to be used in diagnosis of periodontitis with/without clinical examination. The aim of this PhD work was to investigate the presence and levels of various salivary biomarkers of infection, inflammation, and tissue degradation.

This PhD study consisted of salivary specimens from two different study populations: a subsample of the national Health 2000 survey (n = 220) and the Parogene cohort study (n = 445/455). Phospholipid transfer protein (PLTP) activity and salivary concentrations of a chemokine (interleukin (IL)-8), cytokines (IL-1β, -17A, -23, lymphotoxin-α (LTA)), myeloperoxidase (MPO), and bone remodeling-related biomarkers (RANK-ligand, osteoprotegerin, osteocalcin, and osteopontin) were examined. The pre-existing data included clinical and radiographic oral examinations, Cumulative Risk Score (CRS), subgingival bacterial detection, saliva and serum lipopolysaccharide (LPS) activity, saliva and serum antibodies against selected bacteria, glycemic status, risk haplotype of human major histocompatibility complex (MHC) class III, and *LTA* gene polymorphisms.

Salivary PLTP activity was lowest in individuals with advanced tooth loss. LPS activity in saliva was associated with high CRS, while IL-1β, IL-17A, and IL-23 concentrations varied depending on clinical periodontal status. There were associations between salivary IL-8 concentrations and bleeding on probing, probing pocket depth, and the number of teeth, and between MPO concentrations and alveolar bone loss. Bone remodeling-related biomarkers in saliva were either affected by glycemic status or detected at very low levels. CRS associated strongly with subgingival microbial profile and high levels of antibodies against five periodontal pathogens, and additionally, with elevated values of bleeding on probing, probing pocket depth, alveolar bone loss, and number of teeth. MPO was the only biomarker associating with the MHC class III risk haplotype of periodontitis. LTA concentration in saliva associated with *LTA* gene variants.

The results of this PhD study suggest that IL-1β, IL-17A, MPO, and CRS are potential salivary biomarkers to be used in large-scale studies where the aim is to indicate the presence or progression/remission of periodontitis.

KEYWORDS: Periodontitis, saliva, biomarkers, host response, diagnostics

TIIVISTELMÄ

Parodontiitti on hampaan kiinnityskudoksiin vaikuttava infektiolähtöinen tulehduksellinen sairaus. Helposti kerättävä kokosylki on yksi mielenkiintoisimmista näytteistä käytettäväksi parodontiitin diagnostiikan tukena. Tämän työn tavoitteena oli tutkia useiden infektiota, inflammaatiota ja kudostuhoa kuvaavien merkkiaineiden esiintymistä ja pitoisuutta syljessä.

Väitöskirjatyön sylkinäytteet ovat peräisin kahdesta eri tutkimusväestöstä: otos (n = 220) Terveys 2000 -tutkimuksesta ja Parogene-kohorttitutkimuksen aineisto (n = 445/455). Syljestä tutkittiin fosfolipidiä siirtävän proteiinin (PLTP) aktiivisuutta sekä kemokiinin (interleukiini (IL)-8), neljän sytokiinin (IL-1β, -17A, -23, lymfotoksiini-α (LTA)), myeloperoksidaasin (MPO) ja neljän luumetaboliaan liittyvän merkkiaineen (RANK-ligandi, osteoprotegeriini, osteokalsiini, osteopontiini) pitoisuutta. Käytettävissä oli suun kliininen ja röntgenologinen tutkimusdata sekä tiedot yhdistelmämerkkiaineeseen (CRS) perustuvasta parodontaalistatuksesta, subgingivaalisista bakteereista, syljen ja seerumin lipopolysakkaridin (LPS) aktiivisuudesta, syljen ja seerumin tietyistä bakteerivasta-aineista, sokeritasapainosta, ihmisen MHC-kompleksin luokan III riskihaplotyypistä sekä *LTA*-geenin polymorfismeista.

Syljen PLTP-aktiivisuus oli matalin tutkituilla, joilla oli eniten menetettyjä hampaita. LPS-aktiivisuus ja korkea CRS olivat yhteydessä toisiinsa, kun taas kliininen parodontaalistatus liittyi syljen IL-1β-, IL-17A- ja IL-23-pitoisuuksiin. Ienverenvuodolla, taskusyvyyksillä ja hampaiden lukumäärällä oli yhteys syljen IL-8-pitoisuuteen ja MPO-pitoisuudella lisäksi horisontaaliseen alveoliluukatoon. Verensokeritasapaino vaikutti osaan luumetaboliaan liittyvistä merkkiaineista, kun taas osa löytyi hyvin matalina pitoisuuksina. CRS yhdistyi merkittävästi subgingivaaliseen bakteeriprofiiliin ja kohonneisiin parodontopatogeenien vasta-ainepitoisuuksiin, sekä lisääntyneeseen ienverenvuotoon, syviin ientaskuihin, alveoliluukatoon ja hampaiden menetyksiin. Syljen MPO-pitoisuus yhdistyi MHC-kompleksin luokan III riskihaplotyyppiin ja LTA *LTA* geenivariantteihin.

Tämän väitöskirjatutkimuksen tulosten perusteella voidaan esittää, että IL-1β, IL-17A, MPO ja CRS ovat potentiaalisia syljen biologisia merkkiaineita käytettäväksi laajoissa väestötutkimuksissa parodontiitin toteamiseksi.

AVAINSANAT: Parodontiitti, sylki, biomarkkerit, isännän vaste, diagnostiikka

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Abbreviations

ABL   Alveolar bone loss
BMI   Body mass index
BOP   Bleeding on probing
CAL   Clinical attachment level
CEJ   Cemento-enamel junction
COX   Cyclooxygenase
CRS   Cumulative Risk Score
ELISA   Enzyme-linked immunosorbent assay
GCF   Gingival crevicular fluid
GP   Generalized periodontitis
HbA1c   Serum glycated hemoglobin
hBDs   Human β-defensins
HOMA-IR   Homeostatic model assessment-insulin
HPLC   High pressure liquid chromatography
IFMA   Time-resolved immunofluorometric assay
Ig   Immunoglobulin
IL   Interleukin
INF-γ   Interferon-γ
LAL   Limulus amebocyte lysate
LD   Linkage disequilibrium
LOD   Limit of detection
LP   Localized periodontitis
LPS   Lipopolysaccharide
LTA   Lymphotoxin-α
MHC   Human major histocompatibility complex
MMP   Matrix metalloproteinase
MPO   Myeloperoxidase
NK   Natural killer cell
OPG   Osteoprotegerin
PCR   Polymerase chain reaction
PGE_2 Prostaglandin E_2

PLTP   Phospholipid transfer protein
PMN   Polymorphonuclear leukocyte, neutrophil
POC   Point-of-care
PPD   Probing pocket depth
PRR   Pattern recognition receptor
qPCR   Quantitative real-time PCR
RANKL   Receptor activator for nuclear factor κB ligand
ROS   Reactive oxygen species
SNP   Single nucleotide polymorphism
SPSS™   Statistical Package for Social Sciences
TGF-β   Transforming growth factor-β
TIMP   Tissue inhibitor of matrix metalloproteinase
Th   T helper cell
TLR   Toll-like receptor
TNF   Tumor necrosis factor
VDR   Vitamin D receptor

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I   Liukkonen, J., Gürsoy, U. K., Pussinen, P. J., Suominen, A. L. & Könönen, E. Salivary Concentrations of Interleukin (IL)-1β, IL-17A, and IL-23 Vary in Relation to Periodontal Status. *J Periodontol,* 2016, 87: 1484-1491

II   Gürsoy, U. K., Liukkonen, J., Jula, A., Huumonen, S., Suominen, A. L., Puukka, P. & Könönen, E. Associations Between Salivary Bone Metabolism Markers and Periodontal Breakdown. *J Periodontol,* 2016, 87: 367-375.

III   Liukkonen, J., Gürsoy, U. K., Könönen, E., Akhi, R., Salminen, A., Liljestrand, J. M., Pradhan-Palikhe, P., Pietiläinen, M., Sorsa, T., Persson, G. R., Mäntylä, P., Buhlin, K., Paju, S., Sinisalo, J., Hörkkö, S. & Pussinen, P. J. Immunological and Microbiological Profiling of Cumulative Risk Score for Periodontitis. *Diagnostics,* 2020, 10, 560.

IV   Liukkonen, J., Gürsoy, U. K., Könönen, E., Gürsoy, M., Metso, J., Salminen, A., Kopra, E., Jauhiainen, M., Mäntylä, P., Buhlin, K., Paju, S., Sorsa, T., Nieminen, M. S., Lokki, M-L., Sinisalo, J. & Pussinen, P. J. Saliva Biomarkers in Association with Periodontal Parameters and the Periodontitis Risk Haplotype. *Innate Immun,* 2018, 24: 439-447.

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1   Introduction

Periodontitis is a multifaceted biofilm-induced inflammatory disease, which affects tooth-supporting structures, and eventually leads to destruction of periodontal tissue. Periodontitis is traditionally diagnosed with clinical and radiographical examinations. However, in large-scale population studies, this approach may not be feasible because the diagnosis requires trained dental professionals and takes a considerable amount of time. An easy-to-use specimen like saliva for recognizing the disease could be a promising tool for this challenge. Although salivary diagnostics cannot give a site-specific diagnosis of periodontitis, they can provide information on overall periodontal health status and susceptibility factors. In addition, saliva is non-invasively and inexpensively obtained without the need for special equipment or experienced personnel (Miller et al., 2010).

Mainly Gram-negative periodontal pathogens located at the subgingival sites and their biologically active surface structures contribute to the host immune response. Inflammatory immune response plays a key role in pathogenesis and progression of periodontitis (Darveau, 2010). Accumulating knowledge on infection-induced inflammatory responses, especially on cytokine functional networks, has improved our understanding on periodontal pathogenesis and brought potential bacteria- and host-originated biomarkers for salivary diagnostics in periodontology (Preshaw and Taylor, 2011). Among studied biomarker candidates, however, only few are well-established to be suitable for clinical use. Before highly effective diagnostics can be launched to the daily practice, there is a need to confirm a suitable marker, or most probably, a combination of markers (He et al., 2018). Many practical problems (e.g., very low salivary concentrations) and patient-related problems (e.g., the impact of systemic conditions on salivary biomarker levels) need to be solved to utilize periodontal salivary diagnostics (Taylor, 2014).

This study series, evaluating salivary and serum levels of selected biomarkers, was performed to provide further information on suitability of these biomarkers for salivary diagnostics.

2 Review of the Literature

2.1 Structures and functions of the periodontium

Tooth-supporting structures consist of the gingiva, periodontal ligament, root cementum, and alveolar bone. The function of the periodontium is to attach the tooth to the jaw, support teeth against masticatory friction and forces, and to act as a barrier against microbes. In a clinically healthy situation, there are no signs of clinical inflammation (i.e., gingival redness, swelling, bleeding, or exudate), and the periodontium sustains its consistency and structure.

The gingiva is the part of the oral mucosa that conceals the alveolar bone and encircles teeth. The gingival sulcus is the shallow crevice around the tooth between the epithelium lining of free gingiva and the surface of the tooth. Clinically, a probing pocket depth (PPD) of gingival sulcus less than 4 mm is considered as one of the signs of periodontal health (Chapple et al., 2018).

The gingival epithelium can be divided into three different anatomical sections in morphologic and functional point of view; the oral outer epithelium, sulcular epithelium, and junctional epithelium. The structure of the junctional epithelium is highly porous and provides transit passage for neutrophils (also known as polymorphonuclear leukocytes (PMNs)) throughout the epithelium into the gingival sulcus. The junctional epithelium also secretes chemokines and cytokines, and thus has a regulator role in innate immune defense, and it acts as a barrier against constant microbial challenge (Bosshardt and Lang, 2005). Under the epithelium, the gingiva has underlying connective tissue, lamina propria, blood and lymphatic components, and nerves.

The periodontal ligament is composed of complex vascular and highly cellular connective tissue. The most important elements of periodontal ligament are the collagenous fiber bundles inserting to the cementum and alveolar bone (Hassel, 1993). The cementum is a calcified, avascular tissue that forms the outer covering to the root and provides an attaching surface to the periodontal ligament.

The alveolar bone, a tooth-dependent structure, is formed when tooth erupts, and it disappears gradually after the tooth is lost. Bone has a constant physiological remodeling process, and several systemic and local factors affect this process. It is

also notable that the remodeling process has similarities with many aspects of inflammation and repair processes (Sodek and McKee, 2000).

2.2   Periodontal pathogenesis

In a healthy situation, commensal bacteria in dental biofilms maintain a low-level innate immune response, which is essential to the homeostasis of host tissues. An altered microbial composition of subgingival biofilm is an etiological factor of periodontal disease (Darveau, 2010). The host response mounts when the periodontium attempts to eliminate periodontal pathogens. In susceptible individuals, this results in a dysregulated production of inflammatory mediators, such as cytokines, chemokines, prostanoids, and enzymes. In periodontitis, this response fails to clear periodontal pathogens but leads to periodontal tissue destruction (Ebersole et al., 2013).

2.2.1   Resident microbiota and pathogenic biofilms

The mouth is one of the most heavily bacteria-colonized parts of the human body. The relationship between the host and his/her microbiome is dynamic. The balance of this relationship is crucial for oral health. Microbial communities in the mouth have multiple functions including metabolic, physiological, and immunological actions. Many host-related factors, such as smoking and stress, affect the microbiota. In order to properly understand the microbiota, the host and microbes need to be taken as one unit, holobiont (Kilian et al., 2016).

Oral microbes tend to aggregate with each other and adhere to specific surfaces of the oral cavity. They form structurally and functionally organized bacterial communities, biofilms, where they can survive and multiply (Sanz et al., 2017). Periodontal health-associated commensals are important in maintaining the balance of the microbiota. In periodontally healthy individuals, subgingival biofilms seem to be quite stable (Kumar et al., 2006), while shifting of their microbial composition, dysbiosis, associates with periodontitis (Sanz et al., 2017). Qualitative and quantitative alterations with enrichment of certain potential pathogens within subgingival biofilms expose to the initiation of periodontal disease. Nutrients arising from tissue destruction and the anaerobic environment of deepening periodontal pocket together form favorable conditions for inflammophilic periodontal pathogens and pathobionts to grow (Hajishengallis, 2014; Jiao et al., 2014; Herrero et al., 2018).

Traditionally, the color-coded complexes have provided a framework for describing subgingival bacterial ecosystems (Socransky et al., 1998; Socransky and Haffajee, 2005). The model clusters the species highly associated with each other and differentiates the clusters with color codes. The green, purple, and yellow

complexes represent early colonizers. The more pathogenic orange complex and, eventually, the most pathogenic red complex, including *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola*, are facilitated by early colonizers (Socransky et al., 1998). The orange complex with a moderate association with periodontitis contains *Fusobacterium nucleatum*, *Parvimonas micra*, *Prevotella intermedia,* and *Prevotella nigrescens,* and also *Campylobacter rectus* is frequently found in periodontitis individuals (Socransky et al., 1998; Könönen et al., 2007; Teles et al., 2010). Besides the red complex species, *Aggregatibacter actinomycetemcomitans* has been stated to be one of the major periodontal pathogens (Könönen and Müller, 2014).

Host response against pathogens shows individual variability, diminishing the value of their recognition but highlighting the impact of dysbiosis (Lloyd-Price et al., 2016). Red and orange complex bacteria are still considered to have an important role in dysbiotic microbial communities. The development of sequencing techniques and knowledge of microbial communities have emerged new periodontal pathogens, such as *Porphyromonas endodontalis* (Hajishengallis and Lamont, 2012; Pérez-Chaparro et al., 2014) and increased awareness of different roles of known species like *F. nucleatum* and *P. gingivalis.* Traditionally *F. nucleatum* is seen as an important bridging organism (Kolenbrander et al., 2010), but it also enhances oxygen tolerance within biofilms and induces an environmental change in biofilms through hypoxia (Gürsoy et al., 2010b; Mendes et al., 2018).

The virulence of periodontal pathogens is based on their ability to invade periodontal tissues and produce molecules that activate host immune response. Besides bacterial antigens, potential virulence factors include endotoxins like lipopolysaccharide (LPS) of Gram-negative bacteria, exotoxins (e.g. the leukotoxin of *A. actinomycetemcomitans*), and proteolytic enzymes, (e.g., gingipains) (Taylor, 2010). Exposure to bacterial antigens triggers the production of antibodies by the host and binding of these antibodies to bacteria is a hallmark of adaptive immunity. Since the mouth is heavily colonized with bacteria, detectable amounts of antibodies against oral bacteria can also be measured from healthy individuals. Local production of antibodies against periodontal bacteria results in their higher levels in gingival crevicular fluid (GCF) than in serum (Engström et al., 1993; Ebersole et al., 2013).

2.2.2 Inflammatory and immune pathways

An appropriate immune response is important in maintaining periodontal health. Primary defense mechanisms of the host include the epithelium barrier (cell-to-cell junctions, high renewal capability, and antimicrobial peptide production), commensal microbiota (competition with pathogens and maintaining the protective

host response), and saliva and GCF flow (protective enzymes and antibodies) (Schroeder and Listgarten, 1997; Taylor, 2010).

The first stage of host response at the cellular and molecular levels is innate immunity. Cells of the periodontium, such as epithelial cells, fibroblasts, neutrophils, natural killer (NK) cells, and dendritic cells, sense microbial presence through an array of pattern recognition receptors (PRRs) on their surfaces (Mahanonda and Pichyangkul, 2007). Activation of PRRs, e.g., Toll-like receptors (TLRs), leads to the recruitment of specific cells (neutrophils, monocytes), formation of antigen-presenting cells (macrophages, dendritic cells), and activating molecular mechanisms (Garlet et al., 2006; Trombone et al., 2009). Due to the activation of innate immunity, cells of the periodontium initiate to secrete proinflammatory cytokines (e.g., interleukin (IL)-1, -6, -8, and tumor necrosis factor (TNF)-α, growth factors (e.g., epidermal growth factor), proteases (matrix metalloproteinase (MMP) -2, -7, -9, -13), and natural antimicrobial peptides (β-defensins (hBDs), cathelicidin LL-37) (Bosshardt and Lang, 2005). Neutrophils are stimulated by these inflammatory mediators, and their amount is increased on sulcus area (Tonetti et al., 1998). At an inflamed site, neutrophils identify and eliminate bacteria either by phagocytosis or directly through the release of oxidative (myeloperoxidase (MPO)) or enzymatic (serine protease) molecules. Neutrophils use oxidative or non-oxidative mechanisms to kill bacteria within phagosome (Odeberg and Olsson, 1976; Klebanoff, 2005) and MPO is involved in activation of neutrophil elastase proteolytic activity during the process called NETosis (Metzler et al., 2014). Also, monocytes destroy bacteria by phagocytosis (Stuart and Ezekowitz, 2005). Innate immune response is amplified and synchronized by the complement pathway (Damgaard et al., 2015).

If innate immunity is not successful and bacterial challenge is prolonged, an antigen-specific adaptive immune response emerges. Dendritic cells and macrophages perform an important link between innate and adaptive immunity acting as antigen-presenting cells (Théry and Amigorena, 2001; Barker et al., 2002). Adaptive immunity can be divided into two lines: T-lymphocytes mainly associate with cell-mediated and B-lymphocytes with antibody-mediated immunity. Adaptive immune response is highly specific and sustained.

Depending on antigen-presenting interactions and cytokine load, naïve T-cells can differentiate into T helper 1 (Th1) cells, Th2 cells, Th17 cells, Th9 cells, regulatory T-cells (Treg), or unconventional T-cells (γδT cells) (Dutzan et al., 2016). The expression of cell surface molecules (in the gingiva most commonly CD4 or CD8) alters the functions of T-cells. CD4-positive T-cells are Th1 cells that secrete interferon-γ (INF-γ) and promote cell-mediated immunity by activating macrophages and NK cells. CD8-positive Th2 cells secrete IL-4, IL-5, and IL-13, and regulate antibody-mediated immunity and mast cell activity (Mosmann and

Coffman, 1989). Th17 cells secrete IL-17 and IL-22, and they are important in immunity reactions against extracellular bacteria. IL-17 has a number of proinflammatory activities together with IL-1β and TNF-α (Korn et al., 2009). Recent evidence has enhanced the role of Th17 cells in the periodontium as regulators of T-cell response and bone resorption (Campbell et al., 2016). Treg cells help to dampen down immune responses and maintain tolerance against self-antigens, secrete transforming growth factor-β (TGF-β) and IL-10, and have a role in regulating other T-cell subsets (Josefowicz and Rudensky, 2009).

Various developmental stages of B-cells, leading to antibody-producing plasma cells, are also regulated by several cytokines and other factors (Goodnow et al., 2010). Five different classes of antibodies have been identified, including immunoglobulin A (IgA), IgG, IgM, IgD, and IgE (Ebersole et al., 2013). In the field of periodontal research, IgA/IgG are frequently determined. IgG antibodies with long half-life represent the overall exposure to bacteria and are extremely stable in plasma (Papapanou et al., 2004; Lakio et al., 2009). Short half-time IgA antibodies represent recent or continuous exposure to pathogens (Boillot et al., 2016). Plasma cells also contribute to periodontal tissue destruction by producing cytokines, like TNF-α, IL-6, IL-10, and TGF-β. Yet, it is not well understood how different cytokines act in a network in pathogenesis of periodontitis (Preshaw and Taylor, 2011).

2.2.3   Periodontal tissue destruction

Periodontal tissue destruction occurs when phagocyte- and lymphocyte-dominated host responses drive a katabolic activity of periodontal tissues via overexpressed inflammatory cytokines and enzymes (Garlet et al., 2006; Trombone et al., 2009). Due to the presence of cytokines in inflamed tissue, various inflammatory mediators (e.g., prostaglandins) are upregulated resulting in multiple and highly complex feedback loops (Preshaw and Taylor, 2011). After their critical level is reached, degradative mediators (e.g., receptor activator for nuclear factor κB ligand (RANKL) and MMP-8) are produced, leading to the destruction of connective tissue and alveolar bone. At the same time, production of endogenous inhibitors (e.g., osteoprotegerin (OPG) and tissue inhibitors of matrix metalloproteinases (TIMPs)) of the degradative mediators are inhibited. Connective tissue destruction is involved in MMP-8 and MMP-9 predominance caused by neutrophil emigration to inflamed site (Miller et al., 2010). Reactive oxygen species (ROS), like MPO, enhance imbalance between MMPs and TIMPs (Hernández et al., 2011). The osteoclastic effect of RANKL in alveolar bone occurs via its ability to activate osteoclastogenesis by binding to RANK. OPG, instead, neutralizes RANKL and prevents its action. (Ebersole et al., 2013). This manner is not unique for inflammation but part of normal

homeostasis. Namely, the distribution between degradative mediators and their inhibitors ratio has been implicated to pathogenesis of periodontitis (Sorsa et al., 2006; Saint-Pastou Terrier and Gasque, 2017). Prolonged imbalance leads to periodontal tissue destruction, which is seen as a clinical manifestation of periodontitis.
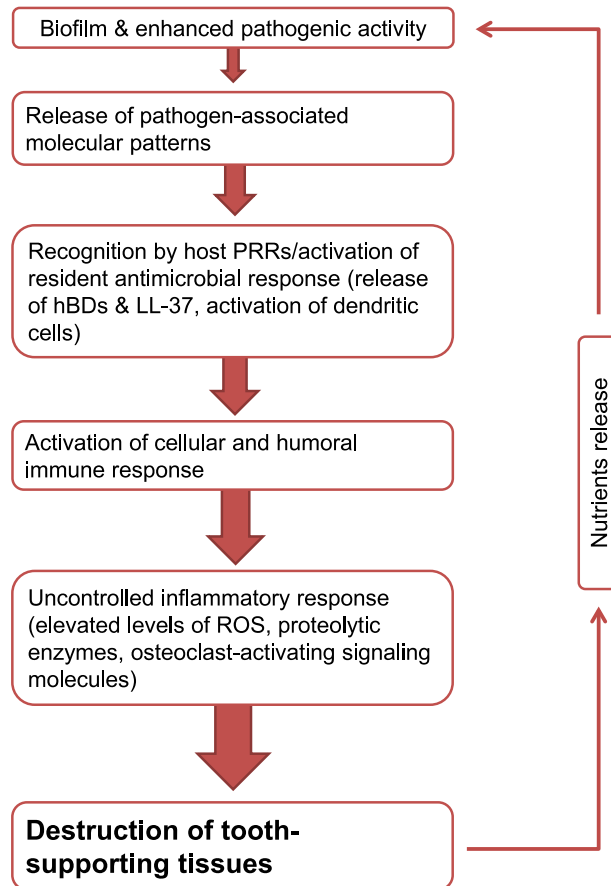
Figure 1. Pathogenesis of periodontal disease. hBDs = human β-defensins, LL-37 = cathelicidin LL-37, PRRs = pattern recognition receptors, ROS = reactive oxygen species.

2.2.4   Risk factors

Several individual-based risk factors have roles in the initiation, progression, and severity of periodontal disease. Smoking, male gender (mainly for behavioral reasons), diabetes, and obesity are well established risk factors by changing individuals' susceptibility or resistance but cannot cause the disease without the emergence of periodontal pathogens. Osteoporosis, stress, and genetic factors are

considered potential risk factors, but more evidence is needed before a clear consensus of their roles can be reached (Genco and Borgnakke, 2013). Although the influence of smoking can be changed via smoking cessation as part of the periodontal treatment, some risk factors, like genetics, are rigid. Modifying the risk factor(s) is usually beneficial for the management of periodontal disease. The current literature suggests bi-directional associations between periodontitis and other chronic inflammatory diseases like diabetes, cardiovascular disease, and rheumatoid arthritis (Pizzo et al., 2010). It has also been estimated that there are common genetic variants, which associate both with periodontitis and with cardiovascular disease (Aarabi et al., 2017).

Smoking is associated with the oral microbial shifting, changes in host response, and impaired wound healing (Johnson and Hill, 2004; Kumar et al., 2011). In 2012, the estimated global age-standardized prevalence of daily tobacco smoking for men was 31.1% and for women 6.2% in the population older than 15 years (Ng et al., 2014). According to the Finnish Health 2000 survey, the percentage of smokers among the males was 29% and among the females 18% (Aromaa and Koskinen, 2004). A meta-analysis (Genco and Borgnakke, 2013) estimated that smokers are at 2.8 times higher risk of developing periodontitis compared to non-smokers, and the risk seems to be dose-dependent.

Diabetes and periodontal disease have a bi-directional relationship. It is assumed that an elevated production of proinflammatory mediators, such as IL-1β, IL-6, and TNF-α, leads to more severe periodontitis and poor diabetes metabolic control. Also bone metabolism is involved in this bi-directional association; bone is acting as an endocrine organ regulating glucose metabolism and, at the same time, hyperglycemia may modulate the RANKL/OPG ratio in periodontal tissues (Ferron et al., 2010; Taylor et al., 2013). Altered balance in bone metabolism, for example reduced bone formation in periodontitis-associated alveolar bone loss, is called uncoupling and is a common finding also in diabetes (Graves et al., 2011). A global comparative diabetes prevalence in 20–79-year-old individuals is estimated to be 9.3% and to grow during following decades (Saeedi et al., 2019). According to the Health 2000 survey, 0.5% of Finns aged 30 or older suffered from type 1 diabetes, and in addition, 4% of males and 3% of females had type 2 diabetes (Aromaa and Koskinen, 2004).

There is evidence that obesity and metabolic syndrome associate with periodontal disease. The exact mechanisms still remain open but possible explanations include the changes in the oral microbiome and chronic systemic inflammatory response (Genco and Borgnakke, 2013). The prevalence of obesity has increased during the past decades. In 2008, a global mean age-standardized body mass index (BMI) was 23.8 kg/m² for men and 24.1 kg/m² for women, and the global age-standardized obesity (BMI $\geq$30 kg/m²) prevalence was 9.8% for men and 13.8%

for women (Finucane et al., 2011). According to the Health 2000 survey, in the population of 30 years or older, 21.2% of males and 23.5% of females were obese (Aromaa and Koskinen, 2004).

Genes regulate the functions of the human body but act differently under different environmental factors (Barros et al., 2018). In chronic diseases like periodontitis, multiple genes play a limited role in progression of the disease, being rather disease-modifying genes than risk variants. Some genes and their variants (polymorphisms) influence the host response to the bacterial load, thus playing part in periodontal disease (Laine et al., 2012). At a genome-wide significance level, few susceptibility gene variants have associated with periodontitis, and the heritability seems to increase with severity of periodontitis (Munz et al., 2019; Nibali et al., 2019; Morelli et al., 2020). A review of Laine et al. (2012) summarized that polymorphisms in genes *IL1, IL6, IL10, vitamin D receptor* (*VDR*), *CD14, TLR14,* and *MMP1* may have an association with periodontitis susceptibility, while a recent meta-analysis (Weng et al., 2016) connected polymorphisms in genes *MMP3, MMP8,* and *MMP9* to periodontitis susceptibility. A certain haplotype within the LTA gene seems to be involved in the susceptibility towards periodontitis (Zupin et al., 2019), and polymorphisms in genes *MMP3*, *CD28*, and *VDR* to initial periodontitis (Heikkinen et al., 2016). Yet, it is not possible to evaluate the impact of multiple genes and polymorphisms on the host response under different environmental conditions (Schaefer, 2018).

Since the genes regulate the host response, it is argued that genetics may influence subgingival colonization in periodontitis (Zhang et al., 2020). Plausible mechanisms relate to changes in genes that are associated with pathways of bacteria recognition or excessive inflammatory environment caused by altered genes, and which favors the growth of specific bacteria. The genes involved in infection, innate and adaptive immune response, and chronic inflammation are carried, for example, in the human major histocompatibility complex (MHC) region located in the chromosome 6. Several inflammation-related proteins, e.g., heat shock proteins, TNF, and lymphotoxins, are in the MHC class III region (Yung Yu et al., 2000). Variations in the MHC region may be important in susceptibility for periodontitis (Palikhe et al., 2008; Folwaczny et al., 2011; Kallio et al., 2014). Locus 6p21.3 in the MHC region has been estimated to have an association with "high periodontal pathogen colonization" traits in genome-wide association studies (Divaris et al., 2012). In a study of Kallio et al. (2014), BAT1-NFKBIL1-LTA risk haplotype was proven to be a significant contributor to the risk of having periodontitis. Bleeding on probing, deepened periodontal pockets of ≥6 mm, and severe periodontitis had the strongest association with this risk haplotype.

2.3 Periodontal disease

Periodontitis is an infection-induced inflammatory disease affecting tooth-supporting structures. Periodontitis affects not only the gingival epithelium and connective tissue under it, but also periodontal ligament and, in particular, alveolar bone. When chronic gingival inflammatory process leads to the activation of osteoclastogenenesis, irreversible changes in alveolar bone occur.

2.3.1 Clinical parameters & traditional diagnostics

The major clinical characteristics of periodontitis are the pocket formation and loss of attachment. Traditionally clinical parameters form a base for a diagnosis of periodontitis and are used in daily practice. Bleeding on probing (BOP) indicates gingival inflammation and active tissue destruction, while PPD $\geq$4 mm indicates a history of periodontal tissue destruction or gingival swelling. Other clinical parameters, clinical attachment level (CAL) and alveolar bone loss (ABL), indicate a history of periodontal tissue destruction, especially alveolar bone destruction. CAL is defined clinically and ABL on radiographs. However, measurements based on marginal radiographic bone loss are not specific enough (Tonetti et al., 2018).

The latest consensus report for the classification for periodontal diseases was published in 2018 (Papapanou et al., 2018). According to this new classification, staging and grading are used to describe the severity of periodontitis and risk for its progression. Certain risk factors, smoking and glycemic status, are used as grade modifiers. A diagnosis of periodontitis is defined primarily when interdental CAL is detectable at $\geq$2 non-adjacent teeth, or buccal or oral CAL $\geq$3 mm with pocketing >3 mm is detectable at $\geq$2 teeth. The new classification also helps to avoid paradox involved in recession of disease severity observed after tooth loss.

The latest classification is the first one defining periodontal health (Chapple et al., 2018). The important change in this new classification is moving from PPD to CAL as the primary clinical parameter of tissue destruction. Although PPD is not the primary clinical parameter, it is still an important part of clinical examination, especially, before the new classification is adopted properly in daily practice.

Figure 2. Periodontal anatomy in health and disease. Left side represents a healthy state. Right side represents periodontitis. ABL = alveolar bone loss, CEJ = cemento-enamel junction, PPD = probing pocket depth.

2.3.2 Epidemiology

There is an increasing global burden of periodontitis (Jin et al., 2016). The occurrence of periodontal attachment loss increases already relatively early in adulthood. The progression accelerates with age. Especially smokers have a risk for progression of attachment loss (Thomson et al., 2013). It has been estimated that mild or moderate periodontitis affects the majority of adults. The global age-standardized burden of severe periodontitis (PPD ≥6 mm or CAL >6 mm) in years 1990–2010 was evaluated to be 11.2% (Kassebaum et al., 2014). On a national level in Finland, according to the Health 2000 survey, 67% of the population of 30 years

or older had at least one periodontal pocket of ≥4 mm (72% of males and 57% of females), and 21% had at least one periodontal pocket of ≥6 mm (26% of males and 16% of females), showing that periodontitis is more common in men than women, and its prevalence is increasing with age (Suominen-Taipale et al., 2008).

2.4 Body fluids for estimating oral health

2.4.1 Saliva as a body fluid

In the context of salivary diagnostics, whole saliva is used to describe combined fluids of the mouth. Whole saliva is a mixture of pure glandular saliva, serum-derived GCF, desquamated oral epithelial cells, other cellular components, micro-organisms and their by-products, and exogenous material (Nieuw Amerongen et al., 2007). Antimicrobial innate defense proteins and peptides of saliva (e.g., lactoferrin, lactoperoxidase, and defensins) protect all oral tissues against microbial attacks (Gorr, 2009). The most important function of saliva is to provide protection against microbes, physical stress, and chemical stress. Besides the protective properties, saliva acts as nutrient source for oral microbes (Marsh et al., 2016). Physical and chemical functions are related to fluidity, including lubrication or clearance, or specific components, such as buffering capacity (Dawes, 2008). There is a large variation in the composition of saliva among individuals and different parts of the oral cavity. Local (e.g., radiation therapy) and systemic (e.g., medications, acquired immune deficiency syndrome, Parkinson's disease, Sjögren's syndrome) factors affect the quality of the salivary functions (Mese and Matsuo, 2007; Grundmann et al., 2009).

2.4.2 Salivary diagnostics in general

Saliva is an advantageous diagnostic specimen. In addition to its local components (e.g., proteins synthesized by acinar cells of salivary glands, bacteria, mucosal cells), saliva contains most of the same molecules that can be found in the systemic circulation, as the serum components of saliva are derived from the local vasculature that originates from the carotid arteries, and thus it may offer even more information than serum (Hofman, 2001; Miller et al., 2010). There is emerging evidence on effects of non-oral diseases (systemic diseases, genetic disorders, immunodeficiency states, and metabolic disorders) on salivary microbial composition; specific microbial consortia may relate to range of systemic conditions widening the diagnostic value of saliva (Acharya et al., 2017). Salivary levels of various medicines reflect the available (unbound to proteins) concentrations in plasma (Tabak, 2007; Lee et al., 2009). Obtaining salivary samples is an inexpensive, non-invasive, patient-friendly, and safe procedure. Handling, storage, and shipping do not require

special arrangements unlike anti-coagulants in blood transfer. Self-collection is also possible (Raju et al., 2019).

Saliva has been shown to be suitable diagnostic medium for many purposes. For example, the detection of several viral infections is possible from a saliva sample (Corstjens et al., 2016). Point-of-care (POC) saliva diagnostics complementary to electrocardiography may provide a rapid screening method for cardiac events for acute myocardial infarction patients (Floriano, 2009). Adenosine deaminase in saliva is an additional diagnostic and prognostic marker for squamous cell carcinoma of tongue (Rai et al., 2011). Especially steroid hormones found in saliva reflect the concentration of unbound steroids in serum. Hormone analysis of saliva has many applications, for example, for doping testing and fertility medicine (Gröschl, 2008). Alcohol and narcotic substances (e.g., amphetamines, cannabinoids, cocaine and opiates) can also be detected from saliva (Bendtsen et al., 1999; Bosker and Huestis, 2009).

In dentistry, saliva tests have been utilized as an aid when assessing individuals´ caries susceptibility by evaluating the presence and amount of lactobacilli and mutans streptococci (Alaluusua et al., 1990), acid production by oral bacteria (Sánchez-Pérez et al., 2009), and oligosaccharides of glycoproteins (Denny et al., 2007)

2.4.3 Gingival crevicular fluid (GCF)

Compared to saliva, GCF is a site-specific oral fluid. GCF is a serum-based fluid originating from the blood vessels of the gingival plexus. It contains components derived from serum, gingival tissues, and subgingival microorganisms (Barros et al., 2016). It is a physiological fluid (transudate) in health and an inflammatory exudate during disease. Like saliva, GCF is considered a potential specimen to detect periodontitis, and biomarker concentrations in GCF are generally higher than those in saliva. The composition of GCF changes during progression of periodontitis and due to the high accuracy even subclinical changes can potentially be detected (Barros et al., 2016). Volumes of GCF samples are relatively small, usually less than 2 µl, and various sampling methods have been reported (He et al., 2018). Collection of GCF is more laborious and has a risk for contamination while on saliva this risk is minimal (Wu et al., 2018). Due to the sensitive collection protocols, the use of GCF is restricted for oral health professionals.

2.4.4 Serum & serum antibodies

In large-scale population studies, clinical periodontal examinations are not always possible to carry out due to practical limitations. If salivary samples have not been included in the study protocol, serum may be used as a specimen to detect individuals

with periodontitis. Many potential salivary biomarkers of periodontitis (e.g., MMP-8, MMP-9, and MPO) can also be found in serum. Systemic conditions, however, may affect serum biomarker levels; for example, statin users have lower MMP-8, MMP-9, and MPO levels in serum than in saliva (Lahdentausta et al., 2018).

It has been shown that LPS can penetrate into periodontal tissues and end up to blood circulation, increasing the risk of systemic problems in periodontitis subjects (Bascones-Martínez et al., 2009). A study performed in mice demonstrated that oral administration of *P. gingivalis* led to increased endotoxin levels in serum, affecting the gut microbiome (Sato et al., (2017). Previous reports indicate that serum LPS correlates with periodontal status and serum LPS activity decreases after periodontal treatment (Pussinen et al., 2004; Paju et al., 2006; Masi et al. 2018).

On one hand, elevated antibody (IgA/IgG) levels against periodontal pathogens in serum of periodontitis patients indicate that they could be used as biomarkers of periodontitis in large-scale population studies (Ebersole et al., 1987; Papapanou et al., 2000; Dye et al., 2009; Pussinen et al., 2011; Kudo et al., 2012; Buhlin et al., 2015; Gadekar et al., 2018). On the other hand, serum antibody levels are mostly related to the carriage of periodontal pathogens, while periodontitis has only a modest effect (Pussinen et al., 2011). Combining salivary and systemic biomarkers in detection of periodontitis has been proposed to overcome this limitation, and the sensitivity and specificity of this approach was demonstrated (Liljestrand et al., 2014; 2018). Nevertheless, both the composition of the oral microbiota and serum antibody response are prone to the presence and severity of endocrine disorders (Akcalı et al., 2014). Thus, the validity and reliability of serum markers for detecting periodontitis under systemic conditions require further studies.

2.5   Potential salivary biomarkers of periodontitis

This section is concentrating on potential salivary biomarkers associated with periodontal disease. Besides IL-1β, TNF-α, IL-6, and RANKL, the markers highlighted by The 7th European Workshop on Periodontology (Kinane et. al., 2011), the section contains markers dealt in **Studies I-IV** (LPS, PLTP, IL-8, IL-17, IL-23, LTA, MPO, OPG, osteopontin, and osteocalcin), and the MMP-TIMP axis.

2.5.1   Agents related to periodontal infection

2.5.1.1   Bacteria & immunoglobulins

Saliva has proven to be a suitable specimen to detect periodontitis-associated bacteria, especially *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, *C. rectus*, and *T. denticola*, in large-scale studies utilizing polymerase

chain reaction (PCR)-based assays (Könönen et al., 2007; Paju et al., 2009). In individuals with periodontitis, there is a correlation between salivary and subgingival levels of specific periodontal pathogens (Haririan et al., 2014), also before and after periodontal treatment (Belstrøm et al., 2018). Salivary microbiota might be a promising tool for evaluating periodontal conditions, and the detection of multiple bacterial species in saliva is superior to that of a single pathogen (Paju et al., 2009; Kageyama et al., 2017; Cieplik et al., 2018).

The odds for pathogen presence can also be evaluated by using serum or saliva IgA/IgG against periodontal pathogens instead of direct detection of those pathogens (Kinane et al., 1999; Plombas et al., 2002; Pussinen et al., 2011; Ebersole et al., 2013). IgA/IgG binding to bacteria in saliva has been studied for *A. actinomycetemcomitans, P. gingivalis, T. denticola, P. intermedia,* and *F. nucleatum* (Sandholm et al., 1987; Hägewald et al., 2000; 2002; 2003; Plombas et al., 2002). In individuals with periodontitis, elevated salivary levels of IgA have been reported against *A. actinomycetemcomitans* (Plombas et al., 2002; Gadekar et al., 2018) and *P. gingivalis* (Plombas et al., 2002), and elevated salivary IgG levels against *A. actinomycetemcomitans* (Gadekar et al., 2018), as well as against *A. actinomycetemcomitans* leukotoxin (Engström et al., 1999).

2.5.1.2 Lipopolysaccharide

Lipopolysaccharide is a surface structure on the outer membrane of Gram-negative bacteria. It is one of the main inducers of the host immune response via TLR activation in periodontal disease. LPS triggers the proinflammatory cytokine production initially via the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, which further activates the mitogen-activated protein kinases (MAPKs) pathways and IκB kinase (IKK) complex (Molteni et al., 2016). In the case of late response, LPS triggers the MyD88-independent pathway to activate the IFN regulatory factor 3 (IRF3) transcription factor, which promotes the type I interferon production. Dendritic cells have a central role in the recognition of LPS and initiation of infection-induced adaptive immune cascade (Kikuchi et al., 2004). Moreover, LPS seems to have a cytokine upregulation effect on several cell types, and it enhances the production of macrophages as an innate immune response. One study showed that IL-23 expression in periodontal ligament cells was induced by *P. endodontalis* LPS (Ma et al., 2017). Another study demonstrated that the production of IL-17 and IL-23 was significantly increased in *P. gingivalis* LPS-treated human periodontal ligament cells (Park et al., 2012). In periodontology probably the most important LPS is that of *P. gingivalis*, a key pathogen of periodontitis (Hajishengallis, 2014). Many studies related to LPS and host response, however, are performed with *Escherichia coli* LPS. Since LPS differs between

different Gram-negative species, also their effects differ from each other (Taylor, 2010; Strachan et al., 2019). Data on the applicability of salivary LPS levels for detection of periodontitis are very limited. Previous studies suggest differences in salivary levels of LPS between individuals with and without periodontal disease when investigating on structural alterations in lipid-A-derived 3-OH fatty acid profiles or using a bioassay for Toll-like receptors 2 and 4 stimulants (Buduneli et al., 2011; Lappin et al., 2011). Moreover, in saliva samples the lipid A chemical structure of LPS differs in periodontitis patients versus healthy individuals indicating increased activity and inflammatory potential of LPS in periodontitis (Mcilwaine et al., 2020). Thus, the regulation of LPS chemical structure seems to have an important role determining whether host bacteria relationship is symbiotic or pathogenic. LPS activity in saliva has been shown to correlate to the number of remaining teeth with a linear trend, but no relation to clinical periodontal parameters has been found (Hyvärinen et al., 2012; Liljestrand et al., 2017).

2.5.1.3   Phospholipid transfer protein

Plasma PLTP plays an important role in plasma lipid metabolism, and it is part of the protein family involved in binding and neutralization of bacterial LPS. PLTP has an ability to promote LPS disaggregation and LPS binding to high density lipoprotein (HDL), resulting in the elimination of LPS from the body (Gautier and Lacrost, 2011; Yu et al., 2016). Since PLTP protects against inflammation triggered by LPS, it can be considered a potential biomarker of Gram-negative bacterial load in periodontal salivary diagnostics. No data exist on the presence or activity of PLTP in saliva. The PLTP activity has been shown to be related to periodontitis in studies, where plasma values were measured before and after periodontal treatment or full mouth tooth extraction (Pussinen et al., 2004; Vuletic et al., 2008).

2.5.2   Markers of inflammation

2.5.2.1   Interleukins

Probably the most studied salivary cytokine to date is IL-1. It includes two isoforms, IL-1α and IL-1β, of which IL-1β is a significant stimulant of bone destruction, being involved in periodontitis (Masada et al., 1990). IL-1β is produced by multiple cells, mainly by fibroblasts, keratinocytes, osteoblasts, macrophages, dendritic cells, monocytes, and leukocytes (Cochran, 2008; Liu et al., 2010; Groeger and Myele, 2015). It is an essential proinflammatory cytokine and, in addition, it induces the synthesis and expression of several other inflammatory mediators. The effects of IL-1β are caused mainly via its ability to enhance the antigen-mediated stimulation of

T-cells (Ben-Sasson et al., 2009). IL-1β also induces various vascular changes during the inflammatory process and regulates neutrophil emigration from the circulation to the periodontium, often in synergy with TNF-α and prostaglandin E_2 (PGE_2) (Taylor, 2014). Furthermore, IL-1β is a potent inducer of bone resorption and connective tissue degradation via its ability to induce MMPs (Birkedal-Hansen, 1993). High concentrations of IL-1β in saliva have been associated with periodontal tissue destruction (Miller et al., 2006; Ng et al., 2007; Tobón-Arroyave et al., 2008; Gürsoy et al., 2009; Fine et al., 2009; Salminen et al., 2014). IL-1β has a very high potential as a salivary biomarker, since its salivary concentrations are not affected by systemic conditions like diabetes or smoking (Jaedicke et al., 2016).

Interleukin-6 is a central proinflammatory cytokine involved in homeostasis and inflammatory pathways. It is produced at early phases of immune responses (Kishimoto, 2005) by a wide range of immune cells, including T- and B-cells, macrophages, and dendritic cells, as well as non-immune cells, including keratinocytes, endothelial cells, and fibroblasts. The production of IL-6 is activated by a number of cytokines, e.g., IL-1β and TNF-α (Van Snick, 1990). IL-6 has an important role in acute inflammation, and it promotes bone resorption (Preshaw and Taylor, 2011). It stimulates MMP secretion of fibroblasts and enhances the inflammatory response to bacterial load in many ways. It also amplifies the inflammatory process and plays a role in T-cell differentiation and the regulation of T-cell balance (Preshaw and Taylor, 2011). The diagnostic performance of IL-6 as a single biomarker is at most acceptable, but its combinatory use, especially with MMP-8, may provide improved diagnostic accuracy (Kc et al., 2020).

Interleukin-8 is a chemotactic cytokine inducing the chemotaxis in neutrophils and their migration to the infection site (Groeger and Meyle, 2015). Oral bacteria induce IL-8 production in gingival epithelial cells through TLR-9 (Kim et al., 2012). Also IL-1β may stimulate the production of IL-8 in oral keratinocytes (Tonetti, 1997) and several other immune and non-immune cells (Commins et al., 2010). Although IL-8 is connected to inflammation, many cross-sectional studies have failed to show an association between salivary concentrations of IL-8 and periodontitis or tissue destruction (Scannapieco et al., 2007; Teles et al., 2009; Rathnyake et al., 2013), however, also opposite reports exist (Kim et al., 2016; Özcan et al., 2016; Kaczyński et al., 2019). Significantly reduced salivary levels have been observed in periodontitis individuals after mechanical periodontal therapy (Sexton et al., 2011; Özcan et al., 2016). A recent meta-analysis by Finoti et al. (2017) reported conflicting evidences regarding IL-8 levels in saliva.

The IL-23/IL-17 axis is one important pathway in an infection-induced inflammatory process. IL-23 is produced by monocytes, macrophages, and dendritic cells in response to bacterial load (Preshaw and Taylor, 2011). It promotes the differentiation of naive CD4+ T-cells into Th17 cells (Iwakura and Ishigame, 2006;

Mills, 2008; Korn et al., 2009). Activated Th17 cells produce IL-17 family cytokines (to date consisting of six members, IL-17A – IL-17F), of which IL-17A is dealt in this work. IL-17A is a proinflammatory cytokine that induces inflammation and bone resorption, stimulating the release of chemokines and expression of matrix metalloproteinases (Beklen et al., 2007; Gu et al., 2013). Various effects of IL-17A are the result of synergy with other cytokines like IL-1β, TNF-α, and INF-γ (Kramer and Gaffen, 2007; Korn et al., 2009; Onishi and Gaffen, 2010; Cheng et al., 2014). There is increasing evidence on the role of IL-17 in immune-mediated inflammatory diseases (e.g., psoriasis, rheumatoid arthritis) (Bunte and Beikler, 2019).

To date, only limited data exist on the role of Th17 cells and IL-17A in the periodontal pathogenesis, and only three studies have reported on the presence of salivary IL-17A. On one hand, IL-17A levels have shown to be lower in generalized periodontitis individuals than in controls (Ozçaka et al., 2011a), and on the other hand, higher levels have been reported in periodontitis individuals than in controls (Awang et al., 2014; Kaczyński et al., 2019). One study found an association between the mean pocket depth and salivary IL-17A concentrations (Gürsoy et al., 2015).

In the majority of recent studies, without further subgrouping, elevated IL-17 concentrations were detected in individuals with periodontitis (Marques et al., 2016; Yang et al., 2016; Batool et al., 2018), but there is also opposing report (Prakasam and Srinivasan, 2014). However, one study has reported low detection frequencies of IL-17 in saliva samples, weakening its potential to be used in salivary diagnostics (Isaza-Guzmán et al., 2015).

No previous data on salivary IL-23 concentrations exist. There is limited amount of conflicting evidence on IL-23 concentrations in GCF samples. One study showed that IL-23 concentrations were proportional to the amount of periodontal tissue damage (Himani et al., 2014), while the other study found relatively high IL-23 concentrations in healthy individuals and lower concentrations in chronic periodontitis individuals without statistical significance (Sadeghi et al., 2018).
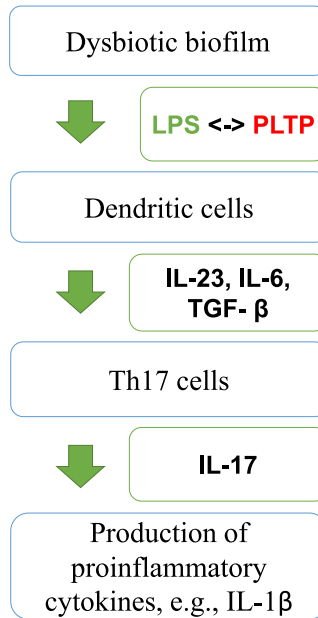
Figure 3.   Schematic picture of inflammatory pathway of periodontitis.

2.5.2.2   Other cytokines

Tumor necrosis factor-α is an essential regulator of immune and inflammatory responses. It is mainly produced by macrophages and T-lymphocytes. Also neutrophils, B-cells, fibroblasts, osteoclasts, and endothelial cells can produce TNF-α. Its proinflammatory effects result from the effects on endothelial cells and their interactions with leukocytes (neutrophil degranulation). TNF-α is also related to vasodilatation and increased vascular permeability via induction of cyclooxygenase (COX)-2 expression in endothelial cells (Pershaw and Taylor, 2011). The effects of TNF-α in the pathogenesis of periodontitis occur via its ability to induce connective tissue destruction and bone resorption (Stashenko et al., 1987). Due to low salivary concentrations of TNF-α, its usability in salivary diagnostics has been questioned (Jaedicke et al., 2016).

LTA (formerly known as TNF-β) is a proinflammatory cytokine involved in the recruitment of inflammatory cells and alveolar bone loss (Assuma et al., 1998). Lymphotoxins are mostly produced by lymphocytes and NK cells (von Boehmer, 1997). Serum LTA concentrations have shown to correlate with deepened periodontal pockets and the number of teeth (Kallio et al., 2014). There are limited data on the link between LTA salivary levels and periodontitis so far. The only available study on salivary LTA concentration demonstrated an inverse relation to the percentage of periodontal pockets (Shyu et al., 2015).

2.5.3   Markers of periodontal tissue destruction

2.5.3.1   Myeloperoxidase

The main role of MPO is to generate hypochlorous acid to kill bacteria. MPO has also an oxidative function to activate proMMPs, especially MMP-8 and -9, to their active forms and inactive TIMPs. (Saari et al., 1990; Spallarossa et al. 2008; Hernández et al., 2011). MPO is mainly released from neutrophils, and its production is induced by host inflammatory mediators like TNF-α, LTA, and IL-1β (Richter, 1990; Saari et al., 1990; Buchmann et al., 2002; Bosshardt and Lang, 2005). In GCF samples, MPO is a highly discriminatory biomarker for site-specific diagnosis of periodontitis (Leppilahti et al., 2014). Elevated levels of salivary MPO have been related to periodontitis (Sakamoto et al., 2008; Meschiari et al., 2013; Nizam et al., 2014; Rathnayake et al., 2015; Lahdentausta et al., 2018), however diagnostic power of salivary MPO was found to be low (sensitivity 64% and specificity 62%; Lahdentausta et al., 2018).

2.5.3.2   Matrix metalloproteinases and their inhibitors

The MMP superfamily consists of over 25 proteolytic enzymes, which can be classified into collagenases, gelatins, stromelysins, membrane-type MMPs, and other MMPs. The effects of MMPs are related to their ability to degrade extracellular matrix components. They have several functions involving the regulation of immune responses and tissue destruction. In periodontal diseases, degradation of soft tissues and organic matrix of the bone are mainly regulated by MMPs (Sorsa et al., 2004; 2006). MMPs are produced by a variety of immune and non-immune cells, including neutrophils, macrophages, monocytes, plasma cells, keratinocytes, fibroblasts, periodontal ligament cells, osteoblasts, and osteoclasts. Several host-related inflammatory cytokines, such as IL-1β and TNF-α, regulate the secretion and activation of MMPs (Sorsa et al., 2006). MMP activation or activity is inhibited by TIMPs and catalytic competence controlled by proenzymes (Sorsa et al., 2004). Collagenase-type MMP-8 (also known as collagenase-2) and gelatinase-type MMP-9 predominate in the pathogenesis of periodontitis. MMP-8 is able to break type I and III collagens, while MMP-9 has ability to degrade type IV and V collagens (Miller et al., 2010). It is well established that elevated salivary MMP-8 and MMP-9 concentrations are associated with periodontitis (Miller et al., 2010; Taylor, 2014), but smoking limits the diagnostic value of MMP-8 by decreasing its salivary levels (Sorsa et al., 2016). Fragmented salivary MMP-8 is a potential but not yet enough studied form of MMP-8 to be used as salivary biomarker (Gürsoy et al., 2018a)

The TIMP family consists of four different proteins (TIMP-1 to TIMP-4). Besides the inhibition of MMPs, TIMPs have several other functions like cell growth promotion, matrix binding, and induction of apoptosis (Brew et al., 2000). Of those, TIMP-1 is most common in salivary diagnostics within periodontology. TIMP-1 is secreted by several non-immune cells (keratinocytes, endothelial cells and fibroblasts) and migratory immune cells (monocytes and macrophages) (Miller et al., 2010). Imbalance in the MMP/TIMP ratio has been implicated to the pathogenesis of periodontitis (Sorsa et al., 2006). Many studies indicate reduced levels of TIMP-1 in saliva of periodontitis individuals or a negative correlation between salivary levels and clinical signs of oral inflammation, but there is some diversity in results. A high MMP-8/TIMP-1 ratio measured from saliva seems to associate with periodontitis or worsened clinical signs of oral inflammation (Hayakawa et al., 1994; Gürsoy et al., 2010a; Isaza-Guzmán et al., 2011; Meschiari et al., 2013; Nizam et al., 2014; Rathnyake et al., 2015).

2.5.3.3   Bone metabolism markers

RANKL and OPG are important bone metabolism-related biomarkers involved in homeostasis and pathologic resorption of alveolar bone. The functions of RANKL in homeostasis are related to bone renewal, optimal T-cell activation, and dendritic cell survival. In the pathogenesis of periodontitis, several proinflammatory cytokines, such as IL-1β and TNF-α, have the ability to activate osteoclastogenesis via upregulating the production of RANKL in activated T-cells and osteoclasts, but also in many other cell types. An increased expression of RANKL stimulates preosteoclasts to mature in active osteoclasts (Preshaw and Taylor, 2011; Ebersole et al., 2013; Saint-Pastou Terrier and Gasque, 2017). Besides bone formation, osteoblasts have an important role in maintaining bone homeostasis through the secretion of OPG, which inhibits the action of RANKL by blocking its binding to its receptor (Saint-Pastou Terrier and Gasque, 2017). Proinflammatory cytokines may inhibit the osteoblast formation (Graves et al., 2011). In the periodontium, OPG is mainly produced by resident connective tissue cells (Belibasakis and Bostanci, 2012). An increased RANKL/OPG ratio is associated with increased osteoclastogenesis and alveolar bone breakdown (Belibasakis and Bostanci, 2012; Saint-Pastou Terrier and Gasque, 2017).

Elevated levels of RANKL in saliva have been associated with periodontitis (Tobón-Arroyave et al., 2012; Tabari et al., 2013; Ochanji et al., 2017; Borges et al., 2019). However, these levels seem to be low, and in some cases even below the detection limit (Frodge et al., 2008). Concerning OPG, equivocal results have been found; while some studies indicated decreased salivary levels in periodontitis individuals (Ramseier et al., 2009; Tabon-Arroyave et al., 2012; Ochanji et al.,

2017), other studies presented increased levels in saliva (Miller et al., 2006; Costa et al., 2010; Al-Sabbagh et al., 2012), and one study did not find any relation to periodontitis (Tabari et al., 2013). A recent meta-analysis by Di Lenardo et al. (2019) failed to show statistically significant associations between salivary OPG levels and periodontitis. The RANKL/OPG ratio, on the other hand, is significantly higher in individuals with periodontitis than in those without (Tobón-Arroyave et al., 2012; Tabari et al., 2013). Moreover, a strong correlation has been found between periodontal disease severity and the RANKL/OPG ratio in saliva (Ochanji et al., 2017). Nevertheless, the RANKL-OPG ratio in GCF samples seems to stay unchanged after non-surgical periodontal treatment (Bostanci et al., 2011), indicating that clinical healing response is not reflected to site-specific levels of these markers. Possible mechanisms behind this observation could be related to continuous RANKL and OPG expressions during periodontal wound healing and remodeling (Baharuddin et al., 2015) or the presence of residual pockets with active disease progression (Borges et al., 2019).

Osteocalcin and osteopontin are non-collagenous calcium-binding proteins of the bone. They form an important component of bone matrix and take part in bone remodeling. Osteocalcin is synthesized by osteoblasts (Booth et al., 2013). During inflammation, proinflammatory cytokines can decrease osteocalcin production in osteoblasts (Graves et al., 2011). Ostepontin is produced by osteoblasts and osteoclasts, while increased levels of osteopontin in inflamed tissues are mainly produced by macrophages and activated T-cells (Denhardt and Noda, 1998). Circulating osteocalcin has been associated with new osteoblasts and bone formation, whereas fragmented osteocalcin has been associated with bone loss (Seibel, 2005). In studies using GCF, the greatest promise for detection of periodontitis appears to be on detecting fragmented osteocalcin (Taba et al., 2005). Osteocalcin levels in GCF have shown to be reduced in individuals with periodontitis (Becerik et al., 2011). There is variation in the results of studies using saliva; an association of elevated osteocalcin concentrations with periodontitis has been found in some studies (Ozçaka et al., 2011b; Miricescu et al., 2014; Betsy et al., 2019), but also negative results have been reported (Bullon et al., 2005). No data exist on salivary osteopontin levels in relation to periodontitis. In one study, it was shown that, before and after treatment with melatonin, changes in the pocket depth correlated with changes in salivary levels of osteopontin (Cutando et al., 2013). In another study, periodontal treatment did not significantly affect the salivary levels of osteocalcin and osteopontin (Beiler et al., 2020). A limited number of GCF-based results are available and they indicate that osteopontin levels are increased in periodontitis individuals (Kido et al., 2001; Sharma and Pradeep, 2006).
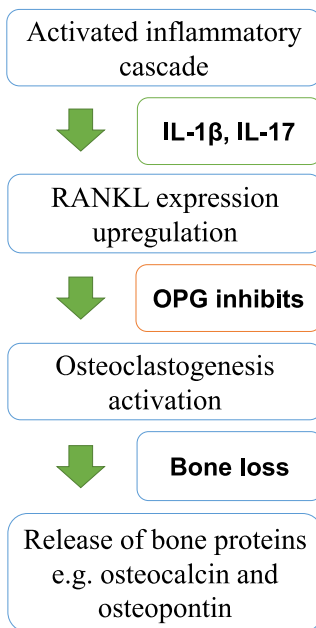
Figure 4. Schematic picture of alveolar bone destruction.

2.5.4 Combinatory use of salivary biomarkers

During the past decades, several biomarkers, such as bacteria and bacterial products, and host-derived agents linked to inflammation and tissue destruction, have been examined and associated with different stages of periodontal conditions (Miller et al., 2010; He et al. 2018). Since the pathogenesis of periodontitis is complicated, it is challenging to define a gold standard to salivary tests in periodontology. Both saliva- and GCF-based results indicate that the usability of a single biomarker varies (Arias-Bujanda et al., 2019; Kc et al., 2020), and that a combinatory use of salivary biomarkers improves diagnostic accuracy. Methods of saliva collection, storage, and analysis vary among studies and restrict assignment of fixed thresholds for salivary biomarker concentrations. Using a combination of salivary biomarkers associated with different stages of periodontal inflammation gives more comprehensive information than a single marker alone (Gürsoy et al., 2011; 2018b).

It has been hypothesized that the nature of chronic periodontitis is a dynamic fluctuating condition (Kinney, 2011). The three main phases of periodontal inflammation are bacterial burden, inflammation, and tissue destruction, following each other in cycles as visualized in **Figure 5**. Due to the fluctuation, a single marker associating with only one phase cannot recognize individuals in other phases of the disease. This weakens the diagnostic value of a single biomarker and encourages a combinatory use of salivary biomarkers. One approach for a combination marker is

Cumulative Risk Score (CRS) developed by Gürsoy et al. (2011). CRS combines the levels of three selected salivary biomarkers, *P. gingivalis*, IL-1β, and MMP-8. These biomarkers represent those three main phases of periodontal inflammation and are individually well-established biomarkers of periodontitis. CRS uses an adaptive study design to distribute the study population into tertiles taking the concentration ranges of selected salivary biomarkers into account. After calculating multiplication of independent biomarker tertiles, individuals are divided into three groups: low, middle, and high risk for having periodontitis. It has been confirmed that CRS associates with periodontitis more strongly than any of the three selected biomarkers alone (Gürsoy et al., 2018b; Salminen et al., 2014).
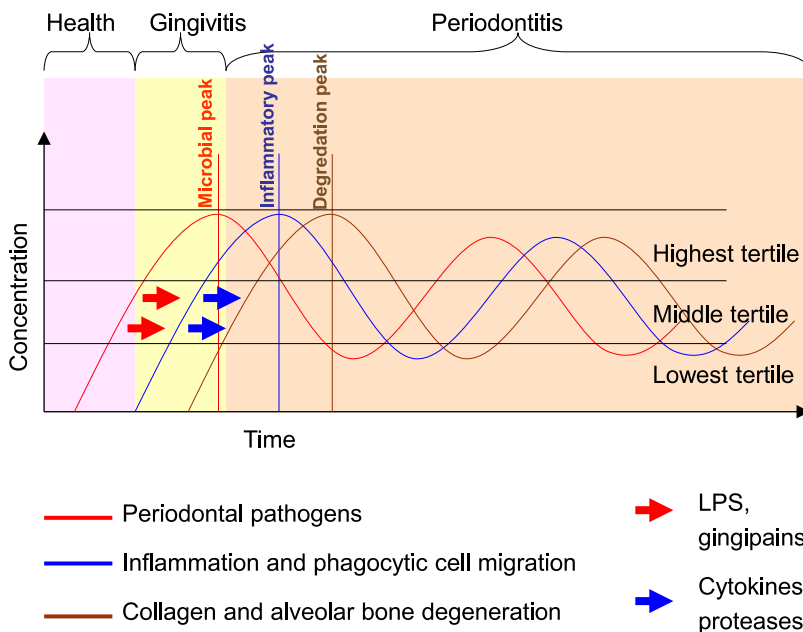
Figure 5. Fluctuating nature of periodontitis. Published in Gürsoy et al., 2011. Reprinted with permission of Hindawi journals.

3   Aims

The hypothesis of the thesis is that selected salivary biomarkers have an association with periodontitis, periodontal parameters, genetic risk of periodontitis, microbial biomarkers or glycemic status.

The overall aim of the study series **(I–IV)** was to investigate salivary concentrations of various biomarkers with an intention to find most potential ones for detection of periodontitis. The specific aims were:

– to evaluate the diagnostic potential of microbial burden-related LPS and PLTP activities (**I, III, IV**), and inflammation-related IL-1β, -8, -17A, -23, LTA (**I, IV**), tissue destruction-related MPO **(IV)**, and bone remodeling-related RANKL, OPG, osteocalcin, and osteopontin (**II**) concentrations in saliva

– to determine whether glycemic status affects the relationship between bone remodeling biomarkers and periodontal status (**II**)

– to profile microbiological and immunological signatures of the Cumulative Risk Score (CRS) via examining its association with saliva and serum antibodies against periodontal pathogens, LPS, and subgingival periodontal pathogens (**III**)

– to examine whether CRS or salivary levels of PLTP activity, LTA, IL-8, and MPO relate to risk haplotype of MHC class III region BAT1-NFKBIL1-LTA (**IV**).

4   Materials and Methods

4.1   Study populations & specimens

4.1.1   Health 2000 survey

Studies I and **II** are based on the subpopulation of 220 individuals, originating from the southern Finland district of Finnish Health 2000 survey, which was carried out by National Public Health Institute (KTL) (currently National Institute for Health and Welfare (THL)). The details of the survey are reported elsewhere (Suominen-Taipale et al., 2008). The individuals were included on the basis of having a salivary sample, recorded periodontal status, at least 20 teeth, and no systemic diseases. Of the 220 individuals, 113 were males and 107 females. Their age varied between 40 to 60 years (mean age 50 years). All individuals of the Health 2000 survey gave a written informed consent and the study protocols were approved by the Ethical Committee for Epidemiology and Public Health of the Hospital District of Helsinki and Uusimaa, Finland.

Clinical oral examinations were carried out by trained dentists and radiographic examinations from digital panoramic radiographs by a specialist of oral radiology. At oral examinations, the deepest measurement for each tooth was recorded as follows: no periodontal pocket, PPD 4-5.5 mm, and PPD $\geq$6 mm. ABL was measured from the mesial and distal surfaces of each tooth and on the furcation areas in mm. The groups describing periodontal status were performed on the basis of PPD $\geq$4 mm as follows: 79 controls with no PPD $\geq$4 mm, 36 subjects having two teeth with PPD $\geq$4 mm (initial localized periodontitis, LP1), 29 subjects having seven teeth with PPD $\geq$4 mm (localized periodontitis, LP2), and 76 subjects having at least 14 teeth with PPD $\geq$4 mm (generalized periodontitis, GP). The radiographic ABL data were used when applying statistical tests with bone remodeling-related biomarkers.

Paraffin-stimulated whole saliva samples were collected on the same appointment before the intraoral examination. Saliva samples were stored at -70°C until used. Then, the selected samples were thawed and centrifuged at 9 300 g for 5 minutes at room temperature. Supernatants were used for salivary biomarker measurements. Details of salivary collection procedures can be found elsewhere (Heistaro, 2008).

Venous blood samples were collected to obtain serum samples. They were allowed to clot for 20 minutes and centrifuged at 1 600–1 800 g for 10 minutes before transferring to the storage tubes. Serum samples were stored at -70°C until used. Detailed methods described elsewhere (Heistaro, 2008)

4.1.2   Parogene study

Studies III and **IV** are based on the subpopulation of the Corogene study, a prospective cohort of consecutive individuals who underwent coronary angiography in Helsinki University Central Hospital (Finland). In this Parogene study, the oral health of the participants was examined. The details of the Corogene and Parogene studies are reported earlier (Buhlin et al., 2011, Vaara et al., 2012). All participants of the Parogene study gave a written informed consent, and the study protocols were approved by the Helsinki University Central Hospital ethics committee, Finland.

In the current PhD work, individuals with saliva determinations (n = 445, 87.6% of the Parogene cohort, **Study III**) or with a salivary sample and genetic information (n = 455, 89.6% of the Parogene cohort, **Study IV**) were included.

Clinical oral examinations were performed by two calibrated periodontists and the radiographic examination from digital panoramic radiographs was carried out by a specialist of oral radiology. For clinical periodontal examinations, PPD and BOP were recorded. Extent of ABL was evaluated radiologically. The Parogene study population included 30 edentulous individuals. Details of oral examination are found elsewhere (Buhlin et el., 2011; Kallio et al., 2014). In **Study III**, the periodontal classification was used only when characterizing the study population and it was based on ABL and PPD as reported earlier by Salminen et al. (2014). In **Study IV**, instead of defining different periodontitis groups, the periodontal parameters BOP, PPD, and ABL were used in statistical analyses.

Paraffin-stimulated whole saliva samples were collected on the same appointment before intraoral examination. Saliva samples were stored at -70°C until used. Then, the selected samples were thawed and centrifuged at 9 300 g for 5 minutes at room temperature. Pellets were used for bacteria measurements and supernatants for all other salivary biomarker measurements. Details of saliva collection are reported earlier (Buhlin et al., 2011).

Venous blood samples were collected to obtain serum samples. The blood-based samples were handled according to the laboratory standards of the Helsinki University Central Hospital and serum samples were stored at -80°C until used (Vaara et al., 2012).

4.2 Collected data

4.2.1 Antibodies against periodontal pathogens

Serum levels of IgA and IgG against whole cell antigens of *A. actinomycetemcomitans, P. gingivalis, P. endodontalis, P. intermedia,* and *T. forsythia* were measured by an enzyme-linked immunosorbent assay (ELISA) (Pussinen et al., 2002; 2011), and details of these analyses are published earlier (Hyvärinen et al., 2012; Liljestrand et al., 2018). Salivary levels of IgA/IgG against the same whole cell antigens were measured by a chemiluminescence immunoassay and details of these analyses are described elsewhere (Akhi et al., 2019).

4.2.2 Cumulative Risk Score (CRS)

The idea of CRS is to identify the individuals with periodontal disease. The calculation of CRS for each individual in a study population is made by dividing the salivary concentrations of *P. gingivalis* (from saliva supernatant), and IL-1β and MMP-8 (from saliva pellets) into tertiles, which are converted to a numerical value of 1, 2, or 3, and then multiplicating the values of each biomarker to a cumulative sub-score (Gürsoy et al., 2011). The final CRS groups formed are as follows: CRS I, indicating the lowest risk (the cumulative sub-scores of 1, 2, and 3); CRS II, indicating the middle risk (the cumulative sub-scores of 4, 6, 8, and 9); and CRS III, indicating the highest risk (the cumulative sub-scores of 12, 18, and 27) of having periodontitis (Gürsoy et al., 2011). The CRS groups used in the current PhD work have been performed and described in detail previously (Salminen et al., 2014).

4.2.3 Genetic risk profiles and *LTA* polymorphisms

Parogene study participants were genotyped for single nucleotide polymorphisms (SNPs). The genetic risk haplotype of MHC class III region BAT1-NFKBIL1-LTA was identified via statistical analyses. Detailed information on the genotyping protocol and constructing the risk haplotype is given elsewhere (Kallio et al., 2014). In **Study IV**, the individuals were further divided as having the risk haplotype in either chromosome or being without the risk haplotype.

The information of *LTA* SNPs in >0.7 linkage disequilibrium (LD) with the rs1041981 was selected for further statistical analyses. These polymorphisms were rs2857708, rs2009658, and rs2844482 (**Study IV**).

4.3 Laboratory analyses

This section, which starts with a table listing the biomarkers and related information, describes the methods used in this PhD work.

Table 1. Summary of the biomarkers used this study. All the laboratory analyses were done blindly.

BIOMARKER	STUDY	SPECIMEN	METHOD	LOD
Subgingival bacteria	III	Pooled subgingival plaque	Checkerboard DNA-DNA hybridization	10^4 cells
LPS activity	I and III	Saliva	LAL assay	N/A
LPS activity	III	Serum	LAL assay	N/A
PLTP activity	IV	Saliva	Lipoprotein independent assay	N/A
IgA and IgG whole cell antigens	III	Saliva	Chemiluminescence immunoassay	N/A
IgA and IgG whole cell antigens	III	Serum	ELISA	N/A
IL-1β	I	Saliva	Luminex	0.4 pg/ml
IL-17A	I	Saliva	Luminex	0.2 pg/ml
IL-23	I	Saliva	Luminex	28.6 pg/ml
LTA	IV	Saliva	Luminex	0.3 pg/ml
IL-8	IV	Saliva	Luminex	0.2 pg/ml
MPO	IV	Saliva	ELISA	0.2 ng/ml
RANKL	II	Saliva	Luminex	4.8 pg/ml
OPG	II	Saliva	Luminex	1.42 pg/ml
Osteocalcin	II	Saliva	Luminex	39.2 pg/ml
Osteopontin	II	Saliva	Luminex	67.8 pg/ml
Insulin	II	Saliva	Luminex	46.3 pg/ml
Insulin	II	Serum	Radioimmunoassay	N/A
Glucose	II	Serum	Hexokinase method	N/A
HbA1c	II	Serum	Immunoturbidimetric method	N/A
CRS	III and IV	Saliva	Combination marker: Luminex + IFMA + qPCR	N/A

ELISA = enzyme-linked immunosorbent assay, IFMA = time-resolvent immunofluorometric assay, LAL = Limulus Amebocyte Lysate, LOD = limit of detection, N/A = not available, qPCR = quantitative real-time PCR

4.3.1 Subgingival bacterial levels

In the Parogene study, pooled subgingival plaque samples were collected from the deepest pocket of each jaw quadrant as described by Mäntylä et al. (2013). Checkerboard DNA-DNA hybridization was used to determine the levels of 29 bacterial species (30 strains). Detailed analyses were performed with the levels of *A. actinomycetemcomitans* (strains Y4 plus ATCC 29523), *P. gingivalis, P. endodontalis, P. intermedia,* and *T. forsythia* (Socransky et al., 2004; Pradhan-Palikhe et al., 2013). Details of 18 Gram-negative species (19 strains) and 11 Gram-positive species, used in analyses, are presented in **Table 2**.

Table 2. Summary of 18 Gram-negative species (19 strains) and 11 Gram-positive species.

SPECIES	GRAM-POSITIVE	GRAM-NEGATIVE
Detailed plus sum analyzed		*Aggregatibacter actinomycetemcomitans* (strains Y4 plus ATCC 29523) *Porphyromonas endontalis** *Porphyromonas gingivalis* *Prevotella intermedia* *Tannerella forsythia*
Only Sum analyzed	*Actinomyces israelii* *Actinomyces naeslundii* *Actinomyces neuii* *Actinomyces odontolyticus* *Parvimonas micra* *Streptococcus constellatus* *Streptococcus gordonii* *Streptococcus intermedius* *Streptococcus mitis* *Streptococcus oralis* *Streptococcus sanguinis*	*Campylobacter gracilis* *Campylobacter rectus* *Campylobacter showae* *Capnocytophaga gingivalis* *Capnocytophaga ochracea* *Capnocytophaga sputigena* *Eikenella corrodens* *Fusobacterium nucleatum* ssp. *naviforme* *Fusobacterium nucleatum* ssp. *nucleatum* *Fusobacterium nucleatum* ssp. *polymorphum* *Fusobacterium periodonticum* *Veillonella parvula* *Treponema denticola*

**Porphyromonas endodontalis* was used only in detailed analyses.

4.3.2 Lipopolysaccharide activity

Before measurements, saliva supernatants were diluted with endotoxin-free water. LPS activity was determined from the serum samples (1:5, vol/vol) and the saliva supernatant (1:40.000, vol/vol) by a commercially optimized Limulus Amebocyte Lysate (LAL) assay coupled with chromogenic substrate (Hycult Biotec, Uden, Netherlands).

4.3.3 Phospholipid transfer protein detection and activity

Saliva PLTP was isolated from saliva samples of six apparently healthy volunteers using heparin affinity column run with a high-pressure liquid chromatography (HPLC) technique. The fractions containing PLTP activity were pooled and applied to a 12.5% SDS-PAGE and visualized by using the monoclonal anti-PLTP antibody, Mab59 followed by a horseradish peroxidase labelled goat-anti-mouse antibody (**Study IV**).

PLTP-facilitated phospholipid transfer activity was measured with a lipoprotein independent assay. The methodology has been described and published earlier (Jauhiainen and Enholm, 2005).

4.3.4 Cytokines, bone remodeling-related biomarkers, and insulin

Salivary concentrations of IL-1β, -8, -17A, -23, LTA, RANKL, OPG, osteocalcin, osteopontin, and insulin were determined by the flow cytometry-based Luminex technology (Luminex, Austin, TX) using the commercially optimized kits (EMD Millipore, Billerica, MA) as recommended by the manufacturer. A detailed description of the Luminex technology can be found elsewhere (Tighe et al., 2013).

4.3.5 Myeloperoxidase

Salivary MPO concentrations were determined by commercially optimized ELISA kits, as recommended by the manufacturer (Immunodiagnostik, Bensheim, Germany).

4.3.6 Serum glucose, insulin, and glycated hemoglobin

Serum-based measurements on diabetes-related biomarkers (glucose, insulin, glycated hemoglobin A1c (HbA1c), and homeostatic model assessment-insulin (HOMA-IR)) were performed in collaboration with National Institute for Health and Welfare. After a minimum of 4 hours fasting, serum glucose concentration was measured with an automated clinical chemistry analyzer (AU400, Olympus, Tokyo, Japan). A radioimmunoassay kit (Phadeseph insulin RIA, Pharmacia, Uppsala, Sweden) was used to measure serum insulin concentrations. An immunoturbidimetric method (Abbot Laboratories, Abbot Park, IL) was used to measure serum HbA1c. The HOMA-IR was used to determine insulin sensitivity by the following formula: (fasting insulin x fasting glucose)/22.5 (Matthews et al., 1985).

4.4   Statistical analyses

A commercial statistical program was used in all studies to analyze the data (SPSS v. 21.0, IBM, Armonk, NY, USA). A part of the salivary markers measured with Luminex did not reach the LOD in some of the samples and the values under detection limit were substituted with LOD/2 (Whitcomb and Schisterman, 2008) (**I, II**), or value 0.01 pg/ml for LTA (**IV**), for statistical analyses. Furthermore, in **Study II,** RANKL and osteocalcin results were not included in the analyses of associations, since the number of samples under LOD was >30% (Uh et al., 2008).

Kolmogerov-Smirnov test and histograms (Chan, 2003a) were used to check the normality of the data distributions. Because of the skewed nature of the data of salivary biomarkers (Chan, 2003b), non-parametric tests (multiple comparisons with Kruskal-Wallis H test and comparisons of differences between two independent groups with Mann-Whitney U test) were used (**I, II**), or logistic regression models were applied (**III**), or values were logarithmically transformed before tests to reach normal distribution, and then parametric tests were used (**III, IV**).

In comparisons, p-values <0.05 (**Study I, II,** and **III**) or <0.01 (**Study IV)** were considered statistically significant.

4.4.1   Characteristics of the study population

The analysis of variance with post hoc pairwise comparisons (Tukey test) was used for following variables: age, number of teeth, number of teeth with PPD $\geq$4 mm, and BMI in comparisons with differences among the study groups (control, LP1, LP2, and GP) (**I, II**). Pearson χ^2 test (Chan, 2003b) was used to compare the percentages of men and smokers among these study groups. T-test was used to compare age, number of teeth, and BMI among the periodontitis risk haplotype groups (**IV**). Joncheere-Tersptra test was used to compare age and number of teeth among the CRS groups (CRS I–III) (**III**). Chi-square test (Chan, 2003c) was used to compare sex, smokers, diabetics, individuals with dyslipidemia and significant coronary artery disease in angiography among the CRS groups or among the periodontitis risk haplotype groups (**III, IV**).

4.4.2   Relationship of salivary biomarkers and different groups

Non-parametric comparisons between two independent groups were performed with Mann-Whitney U test and multiple comparisons with Kruskal-Wallis H test (Chan, 2003b) (**I, II**). Parametric comparisons between two independent groups were performed with t-tests and multiple comparisons with One-Way ANOVA (independent salivary biomarkers) or Chi-square test (CRS) (Chan, 2003b; Chan,

2003c) (**IV**). Jonckheere-Tersptra test was used when examining the relation of CRS groups I–III to serum or saliva IgA/IgG, LPS activity, and subgingival bacterial levels (**III**). Further analysis of the relationship between salivary biomarkers and ABL, age, and serum biomarkers was performed by converting continuous variables to categorical variables (in tertiles) (**II**). Spearman correlation analysis (Chan, 2003d) was used to evaluate correlations between salivary, serum, and periodontal parameters (**I–II**).

4.4.3 Analyses of association

Logistic regression analysis (Chan, 2005) was used to study associations of salivary IL-17A and IL-1β concentrations with different periodontal health state (**I**), salivary osteoprotegerin as tertiles with GP (**II**), high CRS (III vs. CRS I–II) with serum or saliva IgA/IgG and LPS activity, and subgingival bacterial levels (**III**), and salivary biomarkers, including CRS, with the genetic risk haplotype (**IV**). Linear regression model (Chan, 2004) was used to study associations of salivary biomarkers, including CRS, with seven different *LTA* SNPs (**IV**). See the detailed information of adjustments of regression models in **Studies I–IV**.

5 Results and Discussion

5.1 General and methodological aspects

This PhD work consists of four individual studies. which concern the potential of biomarkers in salivary diagnostics from a different point of view. Together, these individual studies test the power and validity of infection, inflammation, and tissue destruction-related biomarkers, starting with their individual use and ending up to their combinational use in detection of periodontitis. Although saliva is the main specimen, serum components and genetic aspects are also included in this PhD thesis. Host response was the main focus in **Study I** where results revealed the role of the IL-23/IL-17 axis at early stages of inflammation as well as reinforced the role of IL-1β as a marker of advanced periodontitis. **Study II** continued with host response, focusing on bone-related biomarkers, and enhanced the hypothesis that glycemic status may influence biomarker concentrations in saliva. **Studies III** and **IV** included a combinatory salivary biomarker CRS besides the individual biomarkers. According to **Study III**, CRS associates strongly with bacterial biomarkers and salivary humoral response against them. In **Study IV**, it was demonstrated that CRS, as well as IL-8 and MPO, associates with periodontal inflammation and tissue destruction.

The relatively large sample sizes (n = 220 in the Health 2000 survey and n = 445/455 in the Parogene study) form one of the major strengths of this PhD work. The age profile of these study populations (40–60 years) was suitable for the study purposes, since the prevalence of periodontitis increases among middle-aged and older individuals. A wide range of information on oral, including panoramic examination, and general health gave a good base to evaluate periodontal status of the study participants. In studies using Health 2000 data (**I** and **II**), the effects of systemic conditions were reduced by selecting non-diabetic individuals. In contrast, in studies using Parogene data (**III** and **IV**), the study population consists of individuals with symptomatic heart disease, and the results may not be fully applicable to healthy individuals.

Lack of a standardized time point of the day for saliva collections, sextant-based measurements of gingival inflammation, and lack of information on clinical attachment levels are among the limitations of this PhD work. In addition, a cross-

sectional study design does not allow us to propose a cause-and-effect relationship between tested markers and parameters nor either estimate the phase of the disease activity of study individuals. On the other hand, large sample sizes and biomarkers representing different phases of the disease process reduce the impact of individual variation of biomarker levels at a certain time point. It is also notable that the genetic information is not bound to any time point.

A methodologically interesting finding of this PhD work was presented in **Study I**. The salivary concentrations of IL-1β were measured previously from the same samples with ELISA (Gürsoy et al., 2009). According to the results, IL-1β concentrations may stay rather constant when stored at -70°C, and besides ELISA, the Luminex technique proved to be suitable to measure this biomarker from saliva. In the literature, there is quite a lot of variation in measuring techniques and LODs between studies. This, combined with different definitions of periodontitis and inability to evaluate individual variations, makes comparisons between previous and current results rather challenging.

5.2 Biomarkers of infection

Lipopolysaccharide produced by Gram-negative periodontal pathogens is one of the main inducers of host immune response in periodontal tissues. In this PhD work, LPS activity was measured from saliva (**I, III**) and serum (**III**) samples by the LAL assay, which is the most sensitive and most widely used method for detecting LPS activity (Manco et al., 2010). It measures biologic activity of a mixture of endotoxins present in saliva or serum instead of evaluating the amount of LPS of specific periodontal pathogens. In **Study I**, LPS did not differ significantly among the three periodontitis (LP1, LP2, and GP) or control groups, and no correlation between LPS activity and tested salivary interleukins (IL-1β, IL-17A, and IL-23) was found. In the literature, there are two studies available on LPS activity in saliva regarding periodontitis or periodontal parameters (Hyvärinen et al., 2012; Liljestrand et al., 2017). According to these studies, salivary LPS activity correlated with the number of teeth and amount of alveolar bone loss in linear trends. In **Study I**, all participants had at least 20 teeth and alveolar bone loss was not included as a clinical parameter, thus it was not possible to correlate salivary LPS activity with these parameters. Therefore, in the absence of confirmatory data, LPS activity may not be considered a suitable biomarker for salivary diagnostics, at least not as the sole parameter, since it seems to reflect rather overall microbial load and not changing directly with periodontal inflammation.

According to our hypothesis, PLTP activity could be another potential biomarker of Gram-negative bacterial load, since it has a protective role against inflammation triggered by LPS. To the author's best knowledge, **Study IV** is the

first to demonstrate that PLTP can be isolated, identified in saliva and that PLTP activity can be measured from saliva samples. Limited data are available regarding PLTP activity in relation to periodontitis. In one study, plasma PLTP activity levels were shown to decrease after periodontal treatment (Pussinen et al., 2004). In other study, plasma PLTP activity was elevated in individuals with periodontitis compared to the reference group (Vuletic et al., 2008). Additionally, plasma PLTP activity inversely correlated with average PPD per tooth, but median PLTP activity did not change significantly after full-mouth tooth extraction. In **Study IV**, measured levels of PLTP activity in saliva were approximately 1/10 compared to those measured from plasma samples (Pussinen et al., 2004), and PLTP activity did not relate to the periodontal parameters (BOP, PPD, and ABL) or to the number of teeth. Lack of relation to periodontal parameters and relatively low salivary concentrations of PLTP activity indicate that it does not tend to be a potential salivary biomarker.

5.3   Biomarkers of inflammation

Interleukin-1β is a well-established salivary biomarker of periodontal inflammation, but also associated with tissue destruction and osteoclast activation. IL-1β was included in the pattern of examined cytokines, because of it's essential role in the inflammation cascade. With this, the aim was to use IL-1β as a reliable reference marker in evaluation of levels of previously untested salivary cytokines, i.e., IL-17A and IL-23. According to the results of **Study I**, salivary concentrations of IL-1β were significantly elevated in the GP group compared to other groups (control, LP1, and LP2) ($p <0.001$). In further analyses (logistic regression models), there was a significant association between the GP group and IL-1β concentrations when age, sex, and smoking were included as confounding variables and the control group was taken as a reference ($p = 0.002$). The results of this PhD work reinforce the view that elevated salivary levels of IL-1β indicate advanced periodontitis rather than being a marker of initial stage of periodontitis. This observation is in line with previous results referred in **Table 3**. Repetitive and systematic changes of IL-1β salivary concentrations among periodontitis groups support its use as a single or combinatory salivary biomarker of periodontitis (Gürsoy et al., 2011; Jaedicke et al., 2016).

Interleukin-8 is an acute phase chemotactic cytokine. In this PhD work, elevated concentrations of IL-8 were observed with increased BOP values (0–25%, 26–50%, and 51–100%) and deepened pockets (PPD <4 mm, 4-5 mm, and ≥6 mm) groups ($p = 0.006$ and $p = 0.001$). In contrast, there was no difference between different ABL groups (no, 1/3, and ≥2/3). Furthermore, elevated salivary concentrations of IL-8 were detected with the increased number of teeth (1–10, 11–20, and 21–32)

(p < 0.001) (**Figure 8**). These findings confirm the role of IL-8 as an acute phase biomarker. The suitability of IL-8 for salivary diagnostics of periodontitis remains unclear, since few periodontal parameters were used in **Study II** and moreover, there are conflicting findings in the literature (**Table 3**). Also, a recent meta-analysis reported conflicting findings regarding IL-8 saliva concentrations, while concentrations in GCF were significantly lower (Finoti et al., 2017). Plausible reasons behind these inconclusive results are methodological or related to the presence of putative inhibitors of IL-8 in saliva (e.g., mucin-like proteins or other large molecules and enzymes).

The hypothesis of **Study I** was that the severity of periodontitis relates to ILs, including IL-17A and IL-23 levels in saliva. IL-23 is linked to IL-17 via its ability to promote the differentiation of naive CD4+ T-cells into Th17, cells which produce IL-17 family cytokines. Of those, IL-17A was selected to be examined in this PhD work. Various effects of it are the result of synergy with other cytokines like IL-1β, TNF-α, and INF-γ (Kramer and Gaffen, 2007; Korn et al., 2009; Onishi and Gaffen, 2010; Cheng et al., 2014). Only few studies have reported the levels of salivary IL-17A in relation to periodontitis, while studies on salivary levels of IL-23 in periodontitis are lacking. Previous reports indicate conflicting findings both for IL-17A and for IL-17 without further subgrouping (**Table 3**). Gürsoy and coworkers (2015) presented an association between mean depth of periodontal pockets and salivary IL-17A concentrations. The findings of **Study I** suggest that salivary levels of IL-17A and IL-23 are connected to localized periodontitis (LP1 or LP2). There was also a slight difference in salivary levels of IL-17A and IL-23 between the control and GP groups. The p-values are presented in **Figure 6**. In further analyses of association, a significant association was found between LP1, LP2, and GP groups and elevated IL-17A concentrations in saliva when age, sex, and smoking were included in the model as confounding variables and the control group was taken as reference (p <0.001, p <0.001, and p = 0.009). However, there were no significant associations between periodontal groups and salivary IL-23. Concentrations of IL-17A and IL-23 correlated weakly but significantly (Spearman ρ: 0.134; p = 0.048).

On the basis of these findings, it can be speculated that the IL-23/IL-17 axis reinforces proinflammatory immune responses at early stages of inflammation. In an immunohistochemical study on gingival biopsies, in which mild, moderate, and severe periodontitis groups were included, an increase in tissue IL-17 levels with the progression of periodontitis was demonstrated (Lester et al., 2007). In contrast, distinct elevation and reduction profiles of IL-17 were not observed, like were done in **Study I**. To the best of authors' knowledge, this study was the first to investigate salivary concentrations of IL-17A and IL-23 in a large study population, where also individuals with localized periodontitis were represented. The results indicate that

elevated levels of IL-17A and IL-23 are limited to the early stages of periodontitis. However, during the analyses, a significant percentage of detected cytokine concentrations remained under LOD: 20% for IL-17A (LOD 0.2 pg/ml) and 61.8% for IL-23 (LOD 28.6 pg/ml). As this phenomenon may affect the results, their usability in salivary diagnostics is restricted. Low salivary concentrations of IL-17 have been demonstrated previously as well (values under LOD 91.3% (LOD 4.2 pg/ml)) (Isaza-Guzmán et al., 2015). Despite the similar profile of these two markers, IL-17A is a more promising candidate for salivary diagnostics than IL-23, since salivary concentrations of IL-17A were higher than those of IL-23. Further research is needed to develop more sensitive laboratory techniques for their detection in saliva samples. This could improve the usability of these two biomarkers in salivary diagnostics of periodontology.

Lymphotoxin-α was studied in **Study IV** to examine its association with the carriage of gene polymorphisms (discussed in section 5.6). LTA is able to induce neutrophils to secrete MPO (Richter, 1990). The literature on LTA is limited and the few available studies present conflicting results in terms of the relation between salivary LTA concentrations and periodontal status (**Table 3**). In this PhD work, salivary LTA concentrations did not relate to BOP, PPD, ABL, or the number of teeth. Furthermore, its levels were relatively low, only 1/100 compared to those measured from serum samples (Kallio et al., 2014), leading to a large percentage of values under LOD (48.4%). Low LTA levels are likely to affect the reliability of the current findings. In future, the development of more sensitive laboratory methods may improve the usability of LTA as a salivary marker in periodontology. However, at present its suitability in salivary diagnostics remains unclear.

Myeloperoxidase is an interesting salivary biomarker, since it has a dual role in the periodontal disease process; on the one side it functions to kill bacteria and on the other side it activates latent proMMPs (Saari et al., 1990; Spallarossa et al., 2008; Hernández et al., 2011). Previous findings, as referred in **Table 3**, suggest that elevated salivary levels of MPO relate to the severity of periodontitis especially via its role in tissue destruction. According to the results of this PhD work, salivary MPO concentrations differed significantly among different groups of BOP (0–25%, 26–50%, and 51–100%), PPD (<4 mm, 4–5 mm, and ≥6 mm) and ABL (no, 1/3, and ≥2/3) values ($p < 0.001$ for all groups) (**Figure 8**). These findings indicate that MPO is a marker of periodontal inflammation and are in line with the literature. It seems that MPO is a potential biomarker for salivary diagnostics in periodontology. The results regarding an association of MPO concentrations in saliva with periodontal risk haplotype are discussed later in this section.

Table 3. Summary of findings in the literature on the presence of the tested inflammatory biomarkers in saliva.

CHANGE IN PERIODONTITIS

REF	SPECIMEN	IL-1B	IL-8	IL-17A	IL-23	LTA	MPO	NOTE!
Miller et al., 2006	Saliva	↑						
Ng et al., 2007	Saliva	↑						
Tobón-Arroyave et al., 2008	Saliva	↑						
Gürsoy et al., 2009	Saliva	↑						
Fine et al., 2009	Saliva	↑						
Salminen et al., 2014	Saliva	↑						
Scannapieco et al., 2007	Saliva		↔					
Teles et al., 2009	Saliva		↔					
Rathnyake et al., 2013	Saliva		↔					
Kim et al., 2016	Saliva		↑					
Özcan et al., 2016	Saliva		↑					
Kaczyński et al., 2019	Saliva		↑	↑				
Ozçaka et al., 2011a	Saliva			↓				
Awang et al., 2014	Saliva			↑				
Prakasam and Srinivasan, 2014	Saliva			↓				No IL-17 subgrouping
Gürsoy et al., 2015	Saliva			↑				Probing pocket depths were taken as independent variable
Isaza-Guzmán et al., 2015	Saliva			↔				No IL-17 subgrouping and only 8.7% over LOD
Marques et al., 2016	Saliva			↑				No IL-17 subgrouping
Yang et al., 2016	Saliva			↑				No IL-17 subgrouping

CHANGE IN PERIODONTITIS

REF	SPECIMEN	IL-1B	IL-8	IL-17A	IL-23	LTA	MPO	NOTE!
Batool et al., 2018	Saliva			↑				No IL-17 subgrouping
Himani et al., 2014	GCF				↑			Periodontal tissue damage was taken as independent variable
Sadeghi et al., 2018	GCF				↔			
Kallio et al., 2014	Serum					↑		Deepened periodontal pockets of 4-5 mm and the number of teeth were taken as independent variables
Shyu et al., 2015	Saliva					↓		Inverse relation with the percentage of periodontal pockets and LTA
Sakamoto et al., 2008	Saliva						↑	
Meschiari et al., 2013	Saliva						↑	
Nizam et al., 2014	Saliva						↑	
Rathnayake et al., 2015	Saliva						↑	
Lahdentausta et al., 2018	Saliva						↑	

↑ = higher concentration; ↓ = lower concentration; ↔ = no change on concentration; GCF = gingival crevicular fluid; LOD = limit of detection

5.4 Biomarkers of bone metabolism

Remodeling of alveolar bone stimulates the release of several bone metabolism-related biomarkers, which eventually end up to saliva. In **Study II**, the concentrations of salivary biomarkers RANKL, OPG, osteocalcin, osteopontin, and insulin were measured. In addition, serum concentrations of insulin, glucose, and HbA1c were measured and HOMA-IR was defined based on insulin and fasting glucose values. According to the hypothesis, insulin and glycemic status may affect the salivary concentrations of bone remodeling-related biomarkers. Glycemic status was presented as a confounding factor on the interactions between periodontal status and bone remodeling-related biomarkers. It was found that salivary concentrations of RANKL, osteocalcin or osteopontin were not related to periodontal status.

Concerning RANKL and osteocalcin, in a remarkable proportion of saliva samples, the detected concentrations remained under LOD or were very low (47.7% and 43.2%, respectively). The low concentration or no detection of RANKL in saliva is in line with an observation by Frodge et al. (2008). Higher levels of osteopontin were found in the middle ABL tertile (3-13 mm of ABL) compared to the highest ABL tertile (> 13 mm of ABL) (p <0.05). Of the tested bone-related markers, OPG was the most promising candidate for salivary diagnostics: only one sample was under LOD and elevated salivary concentrations of OPG were found in LP2 and GP groups compared to controls (p <0.05, see **Figure 6**). In further analyses (logistic regression analysis), elevated OPG concentrations in saliva associated with GP when the age, sex, smoking, and glycemic status were included in the model as confounding variables and the control group was taken as reference (p = 0.03), but statistically significant association was lost when salivary insulin was included in the model.

The literature regarding salivary concentrations of these bone biomarkers is limited (**Table 4**). Before this PhD work, the only available data on osteopontin levels in oral fluids was limited with studies using GCF as a sample material. Based on these studies, elevated osteopontin concentrations have been found in periodontal pockets of periodontitis individuals in comparison to periodontally healthy sites. The results of our study showed higher salivary osteopontin concentrations in middle ABL tertile (3-13 mm of ABL). As seen in **Table 4**, there are conflicting OPG findings in saliva in relation to periodontitis. The present results are in line with those demonstrating elevated salivary OPG concentrations in periodontitis individuals (Miller et al., 2006; Costa et al., 2010; Al-Sabbagh et al., 2012). It is notable, that all tested salivary biomarkers except insulin, differed significantly between genders; the concentrations of RANKL, osteocalcin, and osteopontin were higher and that of OPG was lower in females than in males (p < 0.05). Mechanisms behind these observations remain to be clarified.

None of the tested bone biomarkers works as an ideal salivary biomarker to detect periodontitis. The present work did not consider the RANKL/OPG ratio, which might be more suitable for salivary diagnostics than either one alone (**Table 4**). However, extremely low RANKL levels in saliva remain undetectable in the majority of the cases, limiting the usability of the RANKL/OPG ratio as a salivary biomarker of periodontitis. It may not be feasible to compare the power and validity of biomarkers in different body fluids, since each body fluid carries its own physiological characteristics. Differences in pH, temperature, volume, viscosity, and enzymatic activity are significant determinants in detecting of proteins (Proctor, 2016), in particular, if present at low levels.

In the literature, the controversial results on bone remodeling-related salivary biomarkers are often explained by factors unrelated to bone turnover, such as the clearance and half-life of the indicated biomarker or methodological aspects, but not by glycemic status. Santos and coworkers (2010), using GCF samples, demonstrated that

poor glycemic control in type II diabetic individuals may influence negatively RANKL concentrations and RANKL/OPG ratios before and after periodontal treatment. To the best of the authors' knowledge, **Study II** was the first to evaluate effects of the glycemic status and insulin profile on salivary bone remodeling-related biomarkers. Salivary insulin significantly affected the observed association between OPG and periodontal status. In serum-based studies, OPG concentrations have been shown to increase with the presence of diabetes (Browner et al., 2001) and correlate positively with fasting glucose levels (Shin et al., 2006). Insulin can regulate OPG expression of osteoblasts through insulin receptors, and increased concentrations of insulin inhibit the OPG expression, leading changes in the RANKL/OPG ratio (Ferron et al., 2010). In line with this background, results of this PhD work showed negative correlation between salivary insulin and OPG (Spearman ρ: -0.15; $p < 0.05$). To demonstrate a causal relationship between salivary OPG and insulin levels, a longitudinal study design is warranted.

Besides the pancreas, salivary glands are a potential source of salivary insulin (Kerr et al., 1995). As shown in **Study II**, insulin concentrations in saliva and serum correlated with each other (Spearman ρ: 0.34; $p < 0.001$), indicating that salivary insulin levels are mainly affected by serum insulin levels. Salivary glands can also secrete osteopontin (Obermüller et al., 2006). Osteopontin is involved in the pathogenesis of insulin resistance and type I diabetes (Kahles et al., 2014), and serum insulin and osteopontin levels correlate in obese subjects (Riedl et al., 2008). According to the results of this PhD work, concentrations of salivary insulin and salivary osteopontin correlate significantly (Spearman ρ: 0.74; $p < 0.001$). Furthermore, there was a correlation between insulin resistance values (HOMA-IR) and salivary osteopontin (Spearman ρ: 0.17; $p < 0.05$). These findings indicate that glycemic status significantly affects osteopontin levels in saliva. It has been shown that active osteocalcin promotes the proliferation and insulin secretion of pancreatic beta cells, which eventually leads to the insulin sensitivity (Ferron et al., 2010; Karsenty and Ferron, 2012). In addition, insulin has a direct effect on bone metabolism and circulating biomarkers related to bone metabolism (Ivaska et al., 2015). However, because of low salivary concentrations of osteocalcin and RANKL, it remained unclear whether glycemic status has an effect on these bone markers.

Several pathways are affected by diabetes and insulin, and this PhD work does not describe a cause-and-effect relationship between the tested markers and parameters. It has been shown that advanced glycation end-products and their receptors increase RANKL and decrease OPG synthesis in different cell lines (Taylor et al., 2013). At the same time, they activate the osteoclast formation and activation. Furthermore, in hyperglycemia proinflammatory cytokines IL-1β and IL-6 are increased in periodontal tissues, leading to stimulation of alveolar bone resorption (Taylor et al., 2013).

Table 4. Summary of previous literature of bone remodeling-related biomarkers.

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REF	SPECIMEN	RANKL	OPG	RANK/ OPG RATIO	OSTEO-CALCIN	OSTEO-PONTIN	NOTE!
Frodge et al., 2008	Saliva	↔					Only 19% over LOD
Tobón-Arroyave et al., 2012	Saliva	↑	↓	↑			
Tabari et al., 2013	Saliva	↑	↔	↑			
Ochanji et al., 2017	Saliva	↑	↓	↑			A strong positive correlation of the RANKL/OPG ratio to disease severity
Borges et al., 2019	Saliva	↑					A relatively small n
Miller et al., 2006	Saliva		↑				
Ramseier et al., 2009	Saliva		↓				
Costa et al., 2010	Saliva		↑				
Al-Sabbagh et al., 2012	Saliva		↑				
Bullon et al., 2005	Saliva				↔		GCF osteocalcin concentrations were significantly higher in periodontitis group
Ozçaka et al., 2011b	Saliva				↑		
Miricescu et al., 2014	Saliva				↑		
Betsy et al., 2019	Saliva				↑		
Kido et al., 2001	GCF					↑	
Sharma and Pradeep, 2006	GCF					↑	

↑ = higher concentration/ratio; ↓ = lower concentration/ratio; ↔ = no change on concentration/ratio; GCF = gingival crevicular fluid; LOD = limit of detection

Figure 6. Summary of statistically significant relations of salivary biomarkers and periodontal status (health, localized (LP1 and LP2), and generalized periodontitis (GP)).

5.5 Subgingival bacteria, immunoglobulins and periodontal parameters in association with Cumulative Risk Score

In salivary diagnostics, it is crucial to understand the interactions between the host and microbes. The diversity of the oral microbiome makes it rather impossible to evaluate this interaction in all directions (Wade, 2011). Highly specialized members of biofilm communities can dysregulate the host immune response and cause dysbiosis (Sanz et al., 2017). The presence of multiple periodontopathic species in saliva associate strongly with periodontitis (Paju et al., 2009), as does salivary IL-1β too (Gürsoy et al., 2009).

CRS, a combinatory salivary biomarker, represents the aspects of bacterial burden, host response, and their effects on surrounding tissues. In **Study III**, the ability of CRS to describe the changes in the salivary and subgingival microbiological profile in periodontitis was studied. The levels of all examined subgingival bacteria and bacterial clusters were significantly elevated in individuals with high risk of having periodontitis (CRS group III) ($p < 0.001$). The association with subgingival *P. gingivalis* was expected, but its association with other periodontitis-related bacteria was a novel finding. In further association analyses (logistic regression model), the model being adjusted for age, sex, number of teeth, and subgingival bacterial levels, the sum of subgingival Gram-negative species associated with high CRS (odds ratio (OR) 6.191, 95% confidence interval (CI) 2.098–18.27, $p = 0.001$). These findings indicate that CRS, a combinatory salivary

biomarker, is able to reflect microbial shifts due to periodontitis at subgingival sites. A recent saliva-based study found positive correlations between *Treponema* and *Selenomas* and the tested cytokines (chitinase 3-like 1, sIL-6Rα, sTNF-R1, and gp130/sIL-6Rβ) and a negative correlation between IL-10 and *Filifactor alocis* (Lundmark et al., 2019), confirming the interaction between periodontal pathogens with inflammatory mediators seen in saliva.

Salivary LPS activity is not considered a candidate biomarker for recognizing individuals with periodontitis. The grouping of controls and periodontitis individuals (LP1, LP2, and GP) in this PhD work may have an influence on the present results. However, **Study III** showed that LPS activity in saliva associates with high CRS (OR 2.191 (95% CI 1.384-3.468, $p = 0.001$)) when logistic regression model adjusted for age, sex, number of teeth, and subgingival level of the bacterial species was used. The finding is new and fits with the hypothesis that CRS enhances the possibility to recognize altered bacterial burden related to periodontitis.

In addition to the salivary levels of LPS activity, serum levels were determined in **Study III**. According to Liljestrand et al. (2017), there is a correlation between saliva and serum LPS activity levels, and a significant part of circulating LPS may originate from the oral cavity. In an animal study with mice (Sato et al. 2017), the oral administration of *P. gingivalis* changed the gut microbiome and elevated serum endotoxin levels. Although it has been shown that serum LPS correlates with periodontal status and serum LPS activity is decreased after periodontal treatment (Pussinen et al., 2004; Paju et al., 2006; Masi et al., 2018), **Study III** did not find any association between serum LPS activity and high CRS. This indicates that combining the information on serum LPS activity to CRS does not improve the diagnostic ability of CRS.

In **Study III**, high CRS associated significantly with salivary IgA/IgG antibody levels of *A. actinomycetemcomitans, P. gingivalis, P. endodontalis, P. intermedia*, and *T. forsythia* ($p < 0.001$). Furthermore, the sum of IgA/IgG antibody levels against *A. actinomycetemcomitans, P. gingivalis, P. endodontalis, P. intermedia,* and *T. forsythia* associated with high CRS (for IgA, OR of 7.043, 95% CI 2.252-22.02, $p = 0.001$ and for IgG, OR of 3.788, 95% CI 1.777-8.077, $p = 0.001$), when logistic regression model was used and model was adjusted for age, sex, number of teeth, and subgingival level of the bacterial species. In serum-based analyses, high CRS associated significantly only with the IgA antibody levels of *A. actinomycetemcomitans* ($p = 0.007$)*, P. endodontalis* ($p < 0.001$)*,* and *P. intermedia* ($p = 0.021$) and with the IgG antibody levels of *P. gingivalis* ($p = 0.040$) and *P. endodontalis* ($p < 0.001$). Serum antibody levels were independent from subgingival bacterial levels. It seems that CRS associates more strongly with the local response. This is in line with an observation that serum IgA/IgG levels of *P. gingivalis* and *A. actinomycetemcomitans* mostly relate to periodontal pathogen carriage, and

periodontitis has at most a modest modifying effect to their levels (Pussinen et al., 2011). In comparison to serum, saliva seems to be a more suitable specimen type to recognize biomarkers of active periodontal inflammation. High CRS reflects well IgA/IgG antibody levels against periodontopathic species in saliva supporting the suitability of CRS for salivary diagnostics of periodontitis.

As shown in **Study IV**, higher CRS values associate with determined clinical parameters, including increased BOP, PPD, and ABL values, and the number of teeth ($p < 0.001$). This finding supports the suitability of CRS for large-scale studies for detecting periodontitis and the idea that a combination of biomarkers would work better than any single biomarker alone (Gürsoy et al., 2011; 2018; Salminen et al., 2014).
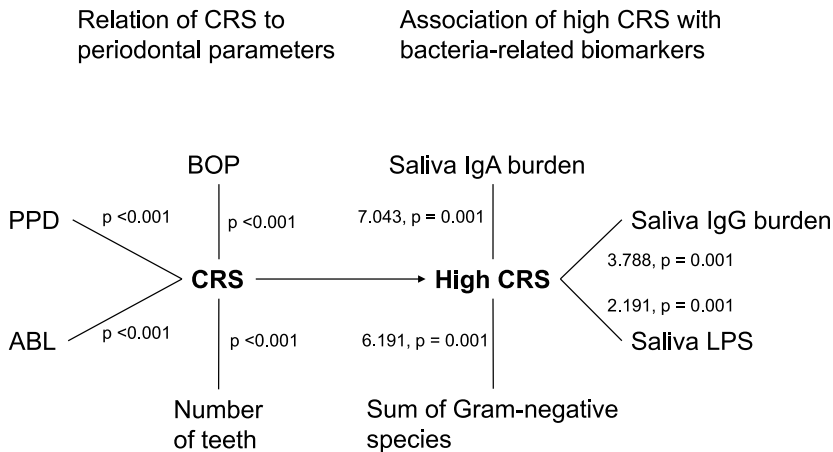
Figure 7. Summary of main findings related to CRS.

5.6 Periodontitis risk haplotype in association with biomarkers

Genomic approaches will allow researchers to characterize behavior of different salivary biomarkers in individuals with different genetic backgrounds (Zeidán-Chuliá et al., 2016). The genes of several inflammation-related proteins, e.g., heat shock proteins, TNF, and lymphotoxins, are carried in telomeric end of human major histocompatibility complex (MHC) class III region (Yung Yu et al., 2000).

The hypothesis of **Study IV** was that PLTP activity, LTA, IL-8, MPO, and CRS appear in saliva at different levels depending on the risk haplotype of MHC class III region BAT1-NFKBIL1-LTA and gene polymorphisms of LTA. In dominant or recessive models of periodontal risk haplotype comparisons, none of the studied biomarkers or CRS differed significantly between the groups. In analysis of

association, where the dominant risk haplotype was selected as an independent variable and model was adjusted with age (Model 1), and additionally with gender, diabetes, and smoking (Model 2), and further with PPD (Model 3) or BOP (Model 4) or ABL (Model 5), MPO associated with increased odds of having periodontitis risk haplotype (OR 1.372 (p = 0.007) in Model 3) per increase of logarithmically transformed unit. Also, salivary PLTP activity, IL-8, and CRS associated slightly in all models with increased odds of having the periodontitis risk haplotype, but these markers did not reach statistical significance. In analysis of association related to *LTA* SNPs, LTA concentrations associated significantly with *LTA* gene variants rs2857708 (p = 0.011), rs2009658 (p = 0.006), and rs2844482 (p = 0.006).

Kallio and coworkers showed that the MHC class III region BAT1-NFKBIL1-LTA risk haplotype (AGCGAC) is a significant contributor to increased risk of periodontitis with the strongest association with BOP, PPD ≥6 mm, and severe periodontitis (Kallio et al., 2014). They also demonstrated that high serum concentrations of LTA relate to periodontal risk alleles of the *LTA* gene. Moreover, it has been shown that the *LTA* +496C variant associates significantly with alveolar bone loss (Palikhe et al., 2008).

The novel findings of **Study IV** were that MPO concentration associated with increased odds for having the risk haplotype and association of LTA with *LTA* gene variants. The polymorphisms of the *LTA* gene seem to have an impact on LTA concentration in saliva, however, the high percentage of values under LOD restricts the liability of current results. LTA can induce the secretion of MPO (Richter, 1990), and it can be speculated that this may be the link between MPO and the risk haplotype. The Parogene study population is rather small to investigate genetic associations and the power calculation was not applicable because some of the salivary biomarkers used were new. Additionally, the study population consists of patients with symptomatic heart disease and thus results may not be fully applicable to a general population, since MHC class III region BAT1-NFKBIL1-LTA may also have a relation to heart diseases (Kallio et al., 2014). Larger general population-based studies are warranted to confirm present novel findings.

Generally, the results of **Study IV** indicate that lymphocytic/systemic salivary biomarkers (PLTP activity and LTA) relate to genetic factors, while local salivary biomarkers (IL-8, MPO, and CRS) seem to have a stronger association with local inflammation and tissue destruction.
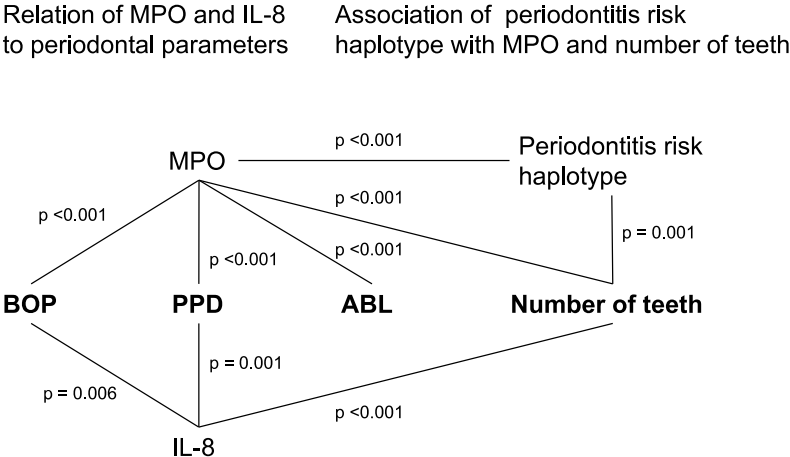
Figure 8. Summary of statistically significant relations of salivary biomarkers and periodontal parameters. Figure also summarizes associations of periodontitis risk haplotype.

6 Conclusions

6.1 Conclusions

The main findings of this PhD thesis can be summarized as follows:

– PLTP activity was detectable in saliva and was the lowest in individuals with advanced tooth loss. Salivary levels of PLTP activity were ten times less than those previously measured from plasma samples. Therefore, PLTP probably plays its main role elsewhere than in the oral cavity, and may not considered a potential salivary biomarker.

– LPS activity in saliva did not relate to periodontal status despite of its association of high CRS. Therefore, its usability as a single biomarker is weak.

– IL-1β was associated with generalized periodontitis, in line with its well-established salivary biomarker of periodontitis. IL-17 has the potential of a salivary biomarker, but its usability warrants further studies. Since IL-23 levels remained under LOD in the majority of the samples, it cannot be considered as a potential salivary biomarker.

– IL-8 concentrations were associated with BOP, PPD, and the number of teeth, and additionally MPO concentration with alveolar bone loss, indicating its potential as a biomarker of periodontal inflammation and tissue destruction.

– Salivary concentrations of bone remodeling-related biomarkers (RANKL, OPG, osteopontin, osteocalcin) are either detected at very low levels or affected by glycemic status. Therefore, their usability as salivary biomarkers of periodontitis is weak.

– CRS is a promising combinatory salivary biomarker for detecting periodontitis in large-scale studies, since it associates strongly with the immunological and microbial profile of the disease.

– LTA concentration in saliva is associated with LTA gene variants, but not with clinical or radiological parameters nor the examined risk haplotype.

- MPO was the only biomarker associating with the MHC class III region BAT1-NFKBIL1-LTA risk haplotype of periodontitis. Underlying mechanisms between MPO and the risk haplotype remain unclear.
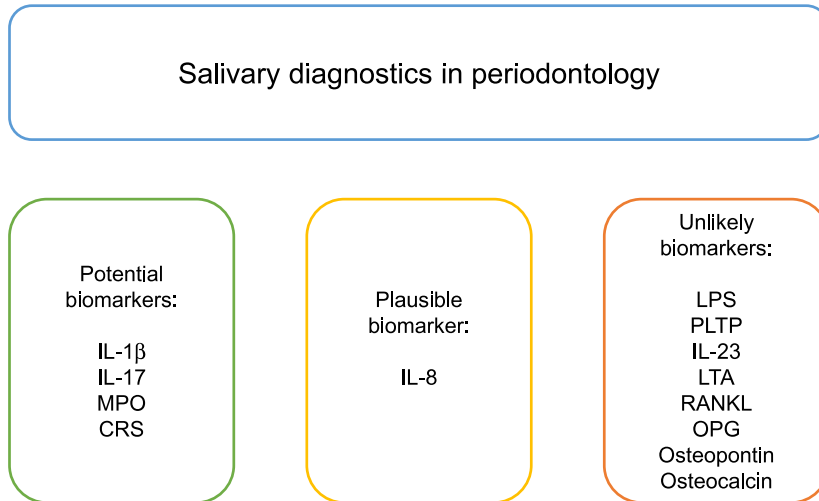
Figure 9. Summary of conclusions of this PhD thesis.

6.2 Future aspects

There is still a limited amount of data on how different conditions and risk factors affect the levels of potential salivary biomarkers, including well-established markers. It has been shown that smoking affects salivary levels of IL-8, MMP-8, MMP-9, TIMP-1, and MPO (Sorsa et al., 2016; Kanmaz et al., 2019; Lahdentausta et al., 2019), and that IL-1β and IL-6 are not affected by smoking (Jaedicke et al., 2016). Although systemic low-grade inflammation is associated with obesity, a recent study by Syrjäläinen et al. (2019) failed to find an association with obesity and IL-1-receptor antagonist, IL-6, IL-8, IL-10, or TNF-α. A review by Jaedicke et al. (2016) stated caution to the conclusions on the impact of systemic diseases on salivary cytokine levels; the amount of data is very limited and none of the reviewed studies was designed specifically to investigate the effect of systemic diseases.

There is an increasing interest in moving towards personalized medicine in periodontology and other fields of dentistry (Belibasakis et al., 2019). Improving the understanding of the complexity of periodontal pathogenesis in relation to several systemic conditions allows determining patient-specific diagnostics. It is expected that in the future salivary diagnostics will have an important role in personalized medicine in periodontics (Ebersole et al., 2015; Korte and Kinney, 2016; Nagarajan et al., 2015; 2017)

This PhD thesis provided evidence on the use of specific biomarkers in saliva for detecting periodontal disease in large-scale studies. Since saliva is an easily collected specimen, it would also be tempting to be used in non-specialized clinical settings. An inexpensive, portable, rapid, and reliable POC salivary diagnostic equipment would launch salivary diagnostics of periodontology in the daily practice (Miller et al., 2010; He et al., 2018; Mitsakakis et al., 2019).

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