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TRANSCRIPTOMIC AND TAXONOMIC PROFILING OF PERIODONTITIS BY MASSIVELY PARALLEL SEQUENCING

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Transcriptomic and taxonomic profiling of periodontitis by massively parallel sequencing
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ABSTRACT

Periodontitis is a microbial-induced, chronic inflammatory disease that affects tissue and bone anchoring the teeth, and can ultimately lead to tooth loss. In addition, periodontitis may contribute to systemic diseases, such as cardiovascular disease (CVD), diabetes, rheumatoid arthritis (RA), and inflammatory bowel diseases. The pathogenesis of periodontitis is complex and involves bacterial products and host-immune response, as well as genetic and environmental factors. Despite extensive studies, the specific genes and mechanisms contributing to disease initiation and progression are poorly understood. The main aim of this thesis was to add to current knowledge of the disease; for this, we generated the transcriptomic and taxonomic profiles from gingival tissue and saliva samples collected from patients with periodontitis and from healthy controls.

Study I explored the gene expression profile of periodontitis compared to the healthy state. Using RNA sequencing (RNA-seq) we characterized the gene expression of gingival biopsies from 62 patients with periodontitis and 62 healthy controls. In patients with periodontitis, RNA-seq showed that genes related to inflammation, the wounding and defence responses, apoptosis, and cell death were up-regulated compared with the controls while genes related to extracellular matrix organization and structural support were down-regulated. The two most highly up-regulated genes in periodontitis were *mucin 4* (*MUC4*) and *matrix metalloproteinase 7* (*MMP7*). Comparisons of the gene expression profiles of periodontitis, CVD, RA, and the inflammatory bowel disease ulcerative colitis (UC) identified only one gene that was commonly up-regulated in all four inflammatory conditions: *pleckstrin* (*PLEK*).

Study II investigated the protein products of *MUC4* and *MMP7* in saliva and gingival crevicular fluid (GCF) samples from patients with periodontitis and from healthy controls. MUC4 levels were significantly lower in the saliva and GCF samples of patients with periodontitis compared to the controls, whereas MMP7 levels were significantly higher. Controlling for age and smoking, analyses revealed a significant association between MUC4 levels and periodontitis. Analysis that controlled for total protein levels, age, and smoking also found a significant association between the ratio of MUC4 to MMP7 levels and periodontitis, suggesting that this combination of MUC4 and MMP7 could possibly be used as a diagnostic marker for periodontitis.

Study III used 16S ribosomal RNA gene sequencing to characterize the taxonomic composition in saliva and investigate the correlation between presence of microorganisms and levels of host inflammatory mediators in samples from 46 patients with periodontitis and 47 healthy controls. The composition of the microbial community differed significantly between these two groups. The microbes *Prevotella* sp., *Phocaeicola* sp., *Fretibacterium* sp., *Streptococcus mitis/parasanguinis*, *Eubacterium saphenum*, *Tannerella forsythia*, *Filifactor alocis*, and *Parvimonas micra* were elevated in the patients with periodontitis, as was the inflammatory mediator glycoprotein 130 (gp130). This mediator, as well as soluble tumour necrosis factor receptor 1 (sTNF-R1), sTNF-R2, soluble interleukin-6 receptor α (sIL-6Rα),

pentraxin 3, and chitinase-3-like 1, were identified as positively correlated with the microbes *Streptococcus* sp., *Selenomonas* sp., *Treponema* sp., and *Selenomonas sputigena*. In contrast, we found the anti-inflammatory mediator IL-10 to be negatively correlated with *Streptococcus* sp., *Eubacterium nodatum*, and *Filifactor alocis*, and positively correlated with *Granulicatella elegans*.

The final two studies of this thesis investigated the patterns of local gene expression in tissue sections. *Study IV* developed a technique for RNA-seq analysis within tissue sections, which *Study V* then used in periodontitis-affected gingival tissue. Differential expression analysis between inflamed compared with non-inflamed areas in the gingival tissue revealed 442 up-regulated genes in the inflamed domain. The three most highly up-regulated genes in the inflamed domain were *immunoglobulin lambda like polypeptide 5* (*IGLL5*), *signal sequence receptor subunit 4* (*SSR4*), and *marginal zone B and B1 cell specific protein* (*MZB1*). In contrast, we identified no down-regulated genes in the inflamed area when compared to the non-inflamed area.

In summary, the studies in this thesis have used massively parallel sequencing to generate transcriptomic and taxonomic characterizations of gingival tissue and saliva samples from patients with periodontitis and healthy individuals in an attempt to identify specific genes involved in the pathogenesis of periodontitis. The thesis also identifies candidate biomarker proteins and microbes for future diagnostic assessment of the chronic inflammatory disease periodontitis.

LIST OF SCIENTIFIC PAPERS

I. **Lundmark A**[*], Davanian H[*], Båge T, Johannsen G, Koro C, Lundeberg J, Yucel-Lindberg T.
Transcriptome analysis reveals mucin 4 to be highly associated with periodontitis and identifies pleckstrin as a link to systemic diseases.
Sci Rep. 2015 Dec 21;5:18475.
[*]Equal contribution

II. **Lundmark A**, Johannsen G, Eriksson K, Kats A, Jansson L, Tervahartiala T, Rathnayake N, Åkerman S, Klinge B, Sorsa T, Yucel-Lindberg T.
Mucin 4 and matrix metalloproteinase 7 as novel salivary biomarkers for periodontitis.
J Clin Periodontol. 2017 Mar;44:247-254.

III. **Lundmark A**, Hu YO, Huss M, Johannsen G, Anderson AF, Yucel-Lindberg T.
The salivary microbiota and its association with inflammatory mediators in periodontitis.
Submitted manuscript.

IV. Ståhl PL[*], Salmén F[*], Vickovic S[+], **Lundmark A**[+], Navarro JF, Magnusson J, Giacomello S, Asp M, Westholm JO, Huss M, Mollbrink A, Linnarsson S, Codeluppi S, Borg Å, Pontén F, Costea PI, Sahlén P, Mulder J, Bergmann O, Lundeberg J, Frisén J.
Visualization and analysis of gene expression in tissue sections by spatial transcriptomics.
Science. 2016 Jul 1;353:78-82.
[*]Equal contribution
[+]Equal contribution

V. **Lundmark A**, Båge T, Jemt A, Mollbrink A, Salmén F, Lundeberg J, Yucel-Lindberg T.
Gene expression profiling of periodontitis-affected gingival tissue by spatial transcriptomics.
Manuscript.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Jemt A, Salmén F, **Lundmark A**, Mollbrink A, Fernández Navarro J, Ståhl PL, Yucel-Lindberg T, Lundeberg J.
An automated approach to prepare tissue-derived spatially barcoded RNA-sequencing libraries.
Sci Rep. 2016 Nov 16;6:37137.

Eriksson K, Lönnblom E, Tour G, Kats A, Georgsson P, Hultgren C, Kharlamova N, Norin U, Jönsson J, **Lundmark A**, Hellvard A, Lundberg K, Jansson L, Holmdahl R, Yucel-Lindberg T.
Effects by periodontitis on pristane-induced arthritis on rats.
J Transl Med. 2016 Nov 3;14:311.

CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
BOP	Bleeding on probing
BZM1	Marginal zone B and B1 cell specific protein
CAL	Clinical attachment loss
CD	Cluster of differentiation
cDNA	Complementary DNA
CVD	Cardiovascular disease
DADA2	Divisive Amplicon Denoising Algorithm 2
dUTP	2'-deoxyuridine 5'-triphosphate
E. saphenum	*Eubacterium saphenum*
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
F. alocis	*Filifactor alocis*
F. nucleatum	*Fusobacterium nucleatum*
GAPDH	Glyceraldehyde 3-phosphate-dehydrogenase
GCF	Gingival crevicular fluid
GO	Gene ontology
Gp130	Glycoprotein 130
H&E	Hematoxylin and eosin
HRP	Horseradish-peroxidase
IBD	Inflammatory bowel disease
IGLL5	Immunoglobulin lambda like polypeptide 5
IL	Interleukin
ISH	*In situ* hybridization
LPS	Lipopolysaccharide
MIP-1α	Macrophage inflammatory protein 1α
MMP	Matrix metalloproteinase
MUC4	Mucin 4
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
P. gingivalis	*Porphyromonas gingivalis*

P. micra	*Parvimonas micra*
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PKC	Protein kinase C
PLEK	Pleckstrin
PMN	Polymorphonuclear leukocyte
PPD	Probing pocket depth
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RDP	Ribosomal Database Project
RIN	RNA integrity number
RNA-seq	RNA sequencing
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
sIL-6R	Soluble interleukin 6 receptor
sPLS-DA	Sparse Partial Least Squares Discriminant Analysis
SSR4	Signal sequence receptor subunit 4
sTNF-R1	Soluble tumour necrosis factor receptor 1
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
TIMP-1	Tissue inhibitor of metalloproteinases 1
TNF-α	Tumor necrosis factor α
t-SNE	t-distributed Stochastic Neighbor Embedding
UC	Ulcerative colitis

INTRODUCTION

In most people, the oral microbiome will at some point in their lifetime cause inflammation. This inflammation is usually short-lived, with the affected tissue recovering to a normal state. In some individuals, however, the inflammation does not resolve and instead becomes chronic. The chronic inflammation of periodontitis, the disease that is the focus of this thesis, perpetuates tissue destruction when left unchecked, ultimately resulting in tooth loss; thus the same host system that protects us is also responsible for the destruction of periodontal tissue. The disease course of periodontitis is “silent”, and patients rarely experience any symptoms before the disease has reached an advanced stage. Severe periodontitis has been linked to heightened systemic inflammation in the body and associated with other chronic inflammatory diseases. Early diagnosis greatly improves clinical outcome, but no biomarkers for early clinical detection of periodontitis have been confirmed. The introductory section of this thesis presents a brief background of the pathogenesis of periodontitis, the association between periodontitis and systemic diseases, the current state of research on biomarkers for periodontitis, and a review of recent studies on the transcriptome of periodontitis and the microbiota involved in this disease.

PERIODONTITIS

Periodontitis is a complex microbial-induced chronic inflammatory disease of the gingiva that destroys tooth-supporting structures, and can ultimately lead to tooth loss (Figure 1). Periodontitis is the most prevalent chronic inflammatory disease in humans today and has probably been common for ~10,000 years (after the domestication of animals and plants that led to a compositional shift in oral microbiota).[1,2] In 2010, almost half of the adult population was affected by periodontitis in some form and its severe form is estimated to affect 743 million people worldwide (nearly 11% of the world population).[3] Periodontitis is not a deadly disease, however, numerous studies have reported indications that it is associated with systemic diseases.[4] Moreover, periodontitis negatively affects the quality of life. Tooth loosening makes chewing more difficult and can therefore have a negative impact on nutrition and diet; aesthetic concerns may adversely affect the patient's social life.[5-8]

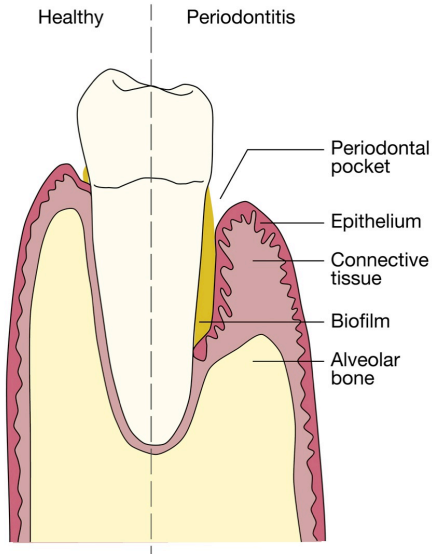

Figure 1. Schematic illustration of healthy periodontal tissue (left) and periodontitis (right). Periodontitis is characterized by degradation of connective tissue and alveolar bone.
Adapted from Yucel-Lindberg and Båge 2013[12], reprinted with permission from Cambridge University Press.

The pathogenesis of periodontitis involves interactions between bacterial products and the host-immune response, which are greatly influenced by genetic and environmental factors (Figure 2).[9] Gingivitis, a common inflammation caused by accumulation of bacteria in the form of dental plaque on the tooth close to the gingiva, always precedes periodontitis. Signs of gingivitis include gingival swelling, redness and bleeding.[10] In healthy individuals, tooth brushing and flossing, which remove the dental plaque, will reverse this condition.[11] Gingival inflammation in individuals who are susceptible for periodontitis however, does not resolve and instead progresses into a chronic inflammation.[12] In this scenario, inflammatory cells and resident cells persistently overexpress inflammatory mediators and proteolytic enzymes, causing the tissue and alveolar bone that support the teeth to degrade.[13]

Clinical outcomes likely depend on a combination of genetic and environmental factors. Genetic factors responsible for regulating inflammation either providing protection or increasing susceptibility, influence the host-immune response. Comorbidities, especially diabetes mellitus and atherosclerosis, may exacerbate the inflammatory state.[5] And environmental factors, including excessive plaque accumulation and smoking, have been reported to influence the disease course.[14-16]

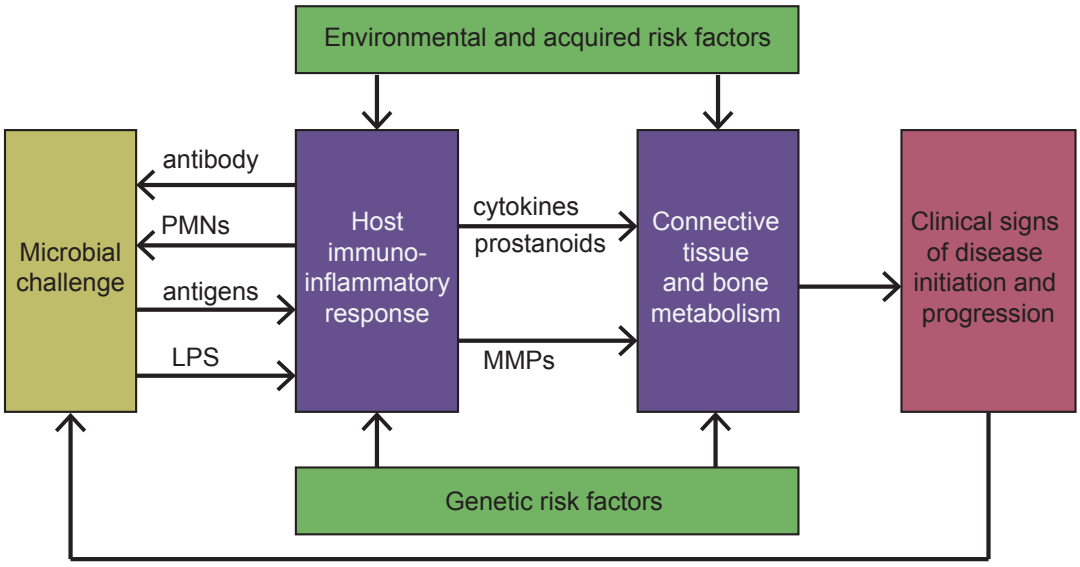

Figure 2. Pathogenesis of periodontitis. Periodontitis results from a complex interaction between the microbial challenge, host immune-inflammatory response and environmental and genetic risk factors. The outcome is destruction of connective tissue and alveolar bone.
LPS, lipopolysaccharide; MMPs, matrix metalloproteinases; PMNs, polymorphonuclear leukocytes.
Adapted from Page and Kornman 1997[6], reprinted with permission from John Wiley and Sons.

Diagnoses of periodontitis are mostly based on clinical evaluation of the soft tissue and bone loss around the teeth, including measurement of probing pocket depth (PPD), clinical attachment loss (CAL), and bleeding on probing (BOP).[5,17] Periodontal treatment initially involves improved dental care and removal of dental plaque by scaling and root planning, whereas more severe periodontitis can be subject to surgery.[5] Because the loss of soft tissue and alveolar bone is largely irreversible, timely and accurate diagnosis is crucial. However, periodontitis is a "silent" disease and in the early stages the patient rarely experiences any symptoms.[5]

Early diagnosis of periodontitis greatly improves clinical outcomes.[5] CAL measurements provide information on past tissue destruction; however PPD, CAL, and BOP do not predict future disease states.[18] Thus, considerable effort has been invested in exploring the use of inflammatory mediators and enzymes as early detection markers for periodontitis. To date, however, no marker has yet been identified as more sensitive than clinical measurements of BOP, PPD, and CAL.[5]

To summarize, periodontitis is a chronic inflammatory disease of the periodontium initiated by microbiota and maintained by the host inflammatory response. Infiltration of the supporting soft tissue and bone by inflammatory cells with the subsequent production of inflammatory mediators causes the destruction of bone and tissue that are vital for tooth support. Upcoming sections will cover these processes in more detail.

Microbial challenge

More than 700 bacterial species have been identified in the oral cavity.[19] These microorganisms can be found both on soft tissue and on teeth were they form biofilms, meaning "a community of microorganisms attached to a surface".[20,21] The most prevalent oral biofilm, dental plaque, exist on tooth surfaces in the form of complex multispecies communities.[21]

The biofilm formation starts with adherence of "early colonizers" that are species with the ability to attach to host-derived glycoproteins, mucins, and other proteins naturally coating the tooth surface.[21,22] The biofilm continues to develop as the late colonizers aggregate to the early colonizing bacteria.[20,21] As the biofilm matures and develop subgingivally, there is also a gradual shift from Gram-positive aerobic bacteria toward Gram-negative and anaerobic species.[16,21,23] Increasing gingival pocket depth changes the gingival environment with respect to pH and oxygen levels, which promotes species favoured by this milieu.[21,24] In addition, the inflammatory response from the host can enrich the environment with inflammatory breakdown products that enhance the growth of certain "inflammophilic" bacteria, which feed off inflammatory products.[25]

Throughout history there have been attempts to identify specific species responsible for periodontitis. In the 1800s, microorganisms were considered as a direct causing agent for periodontal tissue destrucion.[16,26] However, the failure to identify specific pathogens led to the "nonspecific plaque" hypothesis with the assumption that the quantity of dental plaque was the cause of periodontitis, which was the focus of studies between the 1930s and the 1970s.[21,27] This theory was challenged by the identification of certain species associated with diseased sites. A landmark study in 1998 by Socransky *et al.* utilizing a DNA hybridization method identified several bacterial complexes associated with either health or disease. Five major groups of bacteria associated with health or disease were identified and given specific colour designations. Most notably among these complexes was the "red" complex comprising the three species *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, identified as strongly associated with periodontitis.[28] This paradigm has prevailed until recently, partly because of the biasness from the use of culture based methods, which may have overestimated the importance of easily grown species.[25]

Dental plaque associated both with clinically healthy and diseased sites have now been extensively studied. These studies have revealed that the microbiota in dental plaque is much more diverse than previously considered.[29] The current consensus is that a dysbiotic community of microbiota is responsible for the initiation and maintenance of periodontitis. It seems obvious that no single pathogen is causative on its own and that multiple species synergistically form dysbiotic communities contributing to periodontitis. However, it is still not elucidated which specific microorganisms drive the onset and/or development of periodontitis.[16,26,30]

Host response

Although bacteria are considered as the main etiological agent for periodontitis, their presence is not directly responsible for the tissue destruction. It is rather the exaggerated host inflammatory response that causes degradation of periodontal tissues.[31]

Periodontal disease is initiated by a biofilm formation in connection to the gingiva and the subsequent release of bacterial products such as lipopolysaccharide (LPS), peptidoglycans, proteases, and toxins. These substances are recognized by host inflammatory and resident cells equipped with receptors called pattern recognition receptors (PRRs), and an inflammatory response is triggered[12,32,33]. The host inflammatory response is mediated mainly by neutrophils, monocytes/macrophages, and T- and B-lymphocytes, as well as by resident cells in the tissue. These cells release inflammatory mediators, including cytokines, chemokines and proteolytic enzymes, which collectively contribute to destruction of tissue and alveolar bone.[12,13,34-37]

Epithelial cells are the first cells that come into contact with microbial substances. The epithelium functions as a physical barrier against microorganisms and elicits innate and acquired immune responses. Upon stimulation, oral epithelial cells express different pro-inflammatory cytokines and chemokines, including interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumour necrosis factor α (TNF-α), which act as pro-inflammatory signalling molecules.[38-44] Through a series of events, mast cells within the epithelium are stimulated to release vasoactive amines and TNF-α, which contribute to vascular permeability, as well as increased expression of adhesion molecules on endothelial cell surfaces, which allows inflammatory cells that circulate in the blood stream to enter the tissue.[12,32,45] Moreover, dendritic cells take up microbial antigenic products, migrate to the lymph nodes where they encounter T-cells to which they present the antigens.[5,46]

The first inflammatory cells to arrive to the infected tissue are polymorphonuclear leukocytes (PMNs). At the site of infection, PMNs release proteases, such as matrix metalloproteinases (MMPs) and other proteolytic enzymes in order to eliminate microorganisms, but these enzymes also contribute to tissue degradation.[25,47,48] In addition to the production of enzymes, activation of PMN cells also results in synthesis of pro-inflammatory cytokines such as IL-1β and TNF-α, which further enhance the inflammatory response.[47] Moreover, resident cells in the tissue, which are predominantly fibroblasts, respond to the inflammatory milieu and release inflammatory mediators and degradation enzymes, contributing to disease persistence.[49] Lymphocytes, mainly T-cells, and macrophages further invade the tissue and release pro-inflammatory mediators, including IL-1β and TNF-α, as well as proteolytic enzymes such as MMPs. At this stage, the gingival tissue damage is reversible and it is possible for the damaged tissue to heal and remodel. However, in individuals susceptible for developing periodontitis, the inflammation may change character into a B-cell/plasma cell response, with subsequent connective tissue degradation and irreversible bone loss.[12,45,50,51]

ASSOCIATIONS BETWEEN PERIODONTITIS AND SYSTEMIC DISEASES

Periodontitis has been linked to increased systemic inflammation in the body, indicated by raised levels of cytokines and acute-phase markers, such as IL-6 and C-reactive protein, respectively.[16] Although causal links between periodontitis and systemic diseases have not been identified, associations have been reported between periodontitis and diseases such as diabetes mellitus, cardiovascular disease (CVD), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD).

Diabetes mellitus is reported to be the most prevalent systemic disease that increases the risk for periodontitis.[5,52,53] The severity, prevalence, and progression of periodontitis are increased in individuals with diabetes.[53-55] Hyperglycaemia, the hallmark of diabetes, can result in activation of pathways that increase inflammation, oxidative stress and apoptosis.[56,57] Conversely, research suggests also that chronic periodontitis negatively affects metabolic control in individuals with diabetes mellitus.[56-58] Both periodontitis and diabetes mellitus are thought to adversely influence the patient's metabolic balance and overall inflammatory burden.[5]

Numerous cross-sectional, case-control, and epidemiological studies suggest that individuals with periodontitis have increased risk of CVD, independent of confounding factors, such as smoking.[4,59-61] Moreover, clinical intervention studies imply that periodontal treatment reduces systemic inflammation and has positive effects on markers of atherosclerosis.[4,60,62,63] The mechanisms through which periodontitis is associated with CVD are not known. It has been proposed that bacteria translocate from the periodontal pocket into the gingival tissue, enter the circulatory system, and in that way provide an atherogenic stimulus. This is supported by the fact that DNA of periodontal bacteria has been found in human atheromas.[4,64-69] In addition, pro-inflammatory cytokines produced in the periodontium could translocate into the bloodstream, induce an acute-phase response in the liver and thereby promote atherogenesis.[4,70,71] This assumption is supported by the fact that individuals with severe periodontitis have elevated levels of cytokines and acute-phase markers in serum compared to healthy controls without periodontitis. Treatment of periodontitis has also been demonstrated to decrease levels of these systemic inflammatory mediators.[4,60,62]

The association between RA and periodontitis has been assumed based on the similar characteristics of the diseases, which are common chronic inflammatory events and local pathological outcomes.[72] There are conflicting reports on the association between periodontitis and RA, although increasing epidemiological data give support for a link, even after adjusting for common risk factors such as smoking.[4,73-86] RA and periodontitis are hypothesized to be linked to each other through the periodontal pathogen *P. gingivalis*. DNA from *P. gingivalis* has been detected in synovial fluid of up to 80% of RA patients in contrast to healthy controls without RA.[87-89] This periodontal bacterium has the unique capacity of inducing citrullination of proteins. Anti-citrullinated protein antibodies (ACPAs) have been detected in individuals up to fifteen years before clinical onset of RA, and it has been

reported that the levels of ACPAs are correlated with disease severity in individuals with RA.[4,72,84,90]

Research on the association between periodontitis and IBD, including Crohn's disease and ulcerative colitis (UC) indicate that there might be a link between these diseases.[91-97] Similar immunopathogenesis between IBD and periodontal disease has been proposed due to the characteristics of the diseases.[98] Both periodontitis and IBD are multifactorial diseases initiated by bacteria, in which immune system, genetics and environmental factors are involved.[99] Notably, IBD often have oral manifestations, with prevalence between 20-50%, and these oral manifestations can in some cases be present years before onset of IBD.[100-103]

CANDIDATE BIOMARKERS FOR PERIODONTITIS

Clinical measures such as PPD, CAL, and BOP are today the basis for diagnosis of periodontitis. However, the measurement of these parameters requires a trained dental professional, and when these clinical signs appear, the disease has largely progressed to an irreversible stage. Therefore, substantial efforts are being put into identifying biomarkers for early detection of periodontitis.

Potential biomarkers have been investigated in saliva and gingival crevicular fluid (GCF), the exudate that fills the gingival pockets. Saliva is more suitable as a diagnostic fluid than is GCF because it is more easily and non-invasively obtained. Furthermore, GCF reflects inflammatory processes at specific and localized sites, while saliva reflects the inflammatory status of all sites, which is likely to be more clinically relevant.[104]

Proteins that have been investigated as potential biomarkers include markers of inflammation (IL-1β[105-108], IL-6[109-114], macrophage inflammatory protein 1α [MIP-1α][115,116], prostaglandin E2 [PGE2][117], and TNF-α[118-120]), markers of connective tissue degradation (MMP8[118,120-123], MMP9[116,121,123,124], tissue inhibitor of metalloproteinases 1 [TIMP-1][107,120,124], aspartate aminotransferase [AST][122,125-130], and alkaline phosphatase [ASP][119,126,127,130]), as well as markers of alveolar bone turnover/resorption (osteoprotegerin [OPG][116,119,121,123,131] and carboxyterminal telopeptide of type I collagen [CTX][116,120,121,123,132]). Moreover, microbial pathogens have been suggested as potential biomarkers for periodontitis (*Aggregatibacter actinomyctemcomitans*[115,120], *P. gingivalis*[107,121,123,126,133], *Prevotella intermedia*[126,133], *T. forsythia*[107,121,123,133], and *T. denticola*[121,123,133]).[134]

Recently, a meta-review focusing on the sensitivity and specificity of reported host-derived potential biomarkers was published.[135] In this review the authors identified one salivary biomarker, MIP-1α, that could discriminate between periodontitis and health with high accuracy.[116] In addition, the meta-analysis identified two biomarkers, IL-1β and IL-6, to have acceptable accuracy for the assessment of periodontitis.[117,136] However, the authors of the meta-review concluded that these findings need to be validated by additional larger studies.[135]

When IL-1β and IL-6 were analysed in combination with each other or together with MMP8, they had better accuracy in discriminating periodontitis and health than when analysed alone.[136] These findings suggest that a panel of biomarkers may have better diagnostic utility than single markers. Moreover, some studies have reported promising results on the combination of host-derived and bacterial markers as diagnostic tools for periodontitis.[121,123,137,138] For instance, Salminen *et al.* (2014) reported on the salivary levels of MMP8, IL-1β, and *P. gingivalis*. The levels of these three markers were combined into "cumulative risk scores", which demonstrated strong association with periodontitis.[138] MMP8 is also today the only biomarker commercially available, in some European countries.[5,139]

TRANSCRIPTOME ANALYSIS OF PERIODONTITIS

Techniques for gene expression analysis

Genome-wide transcriptome analysis is now routine thanks to technical advances during the last decades. High throughput analysis of whole transcriptomes first became possible with microarrays, which are still being used in many laboratories, although now being largely replaced by massively parallel sequencing. Genome-wide transcriptome studies, as performed by microarrays or massively parallel sequencing, are usually carried out for exploratory analysis as a guidance for subsequent analysis. For studies where the genes of interest are already identified, targeted approaches for gene expression, such as reverse transcription quantitative polymerase chain reaction (RT-qPCR), or *in situ* hybridization (ISH), are commonly performed.

Microarray technology was the first high-throughput method for gene expression analysis. Microarrays consist of clusters of DNA probes immobilized in discrete locations on solid substrates, for instance glass slides. Fluorescently labelled complementary DNA (cDNA) derived from transcripts can hybridize to the array and the intensities of the fluorescent signals are used to determine the amount of bound cDNA.[140] While microarrays allow for analysis of whole transcriptomes, the sequences need to be known beforehand for design of the probes on the microarray.[141]

Massively parallel sequencing approaches (also called next generation sequencing) were introduced in the early 2000s and their use has steadily grown since then, due to increase in throughput and decrease in costs.[142] Today, Illumina is undoubtedly the largest sequencing platform on the market. The technology uses a sequencing-by-synthesis approach where fluorescently labeled nucleotides are added sequentially to clusters of single stranded DNA molecules. The sequence of bases is deciphered by recording the fluorescent signals.[143] Compared to microarrays, massively parallel sequencing has a greater dynamic range, higher sensitivity and specificity, and it has the ability to detect novel transcripts.[141,144-147]

Most studies on tissue transcriptomes have been performed on RNA extracted from whole biopsies, thus producing data that represent averages of the gene expression across all cells

within the biopsies. Therefore, several single-cell RNA sequencing (RNA-seq) technologies have been developed with optimization of library preparation protocols to handle less input material.[148-151] Using these techniques new insights into development[152,153], cancer[154,155], neuroscience[156,157], and inflammation[158,159] have been gained. However, while these methods can reveal cell-to-cell variability, they require tissue dissociation, which causes loss of positional information. For that reason, spatially resolved gene expression methods that add information on the localization of cells within tissue have been developed. For instance, laser capture microdissection has been used for dissecting cells or small anatomical regions from tissue sections followed by RNA-seq, using the single-cell RNA-seq methods describe above.[160-162] Moreover, ISH techniques have been optimized to simultaneously target hundreds of different transcripts, down to single molecule resolution.[163-167] Several additional techniques have been proposed, including sequencing directly in tissue sections and cells, although most of these methods are still at a proof-of-concept stage.[168,169]

Capturing the transcriptome in its entirety, while at the same time preserving the exact spatial information of all transcripts, remains a distant goal for the above-mentioned techniques. However, considerable resources have been invested towards this goal and the resolution is increasing. In *Study IV* of this thesis, we propose a novel method for transcriptome-wide analysis of tissue sections with spatial resolution.

Previous studies on periodontitis

The global transcriptome profiles of periodontitis-affected gingival tissues in comparison to healthy gingival tissues have previously been investigated in a few studies. Most of these studies have been performed using microarrays, while two more recent studies employed RNA-seq for interrogating the transcriptome in periodontal tissues.

In 2008, Papapanou and co-workers compared gene expression profiles of gingival biopsies from diseased and healthy sites in 90 patients with periodontitis. Their results demonstrated that differentially expressed genes were mainly related to apoptosis, antimicrobial humoral response, antigen presentation, regulation of metabolic processes, signal transduction, and angiogenesis.[170] The same group also investigated the transcriptomes of gingival tissues in chronic and aggressive periodontitis, the two major forms of periodontitis. The differences in gene expression between the two groups were small. However, when they in another study applied a model-based clustering approach to the same data, they could identify two subgroups based on the transcriptome data instead of on the clinical parameters. The gene expression in the first subgroup was primarily related to cell proliferation while the second subgroup was associated with lymphocyte activation and unfolded protein responses.[171,172]

The above-mentioned studies employed microarrays for the transcriptome analysis, and our group was the first to report the gene expression in periodontitis using RNA-seq technique. Twelve patients with periodontitis were included and two biopsies from each patient were collected – one from a periodontitis-affected site and one from a healthy site. The results

revealed that periodontitis-affected sites demonstrated an up-regulation of inflammatory pathways, compared to healthy sites.[173]

In 2014, Becker and colleagues also used RNA-seq to investigate the transcriptome in periodontitis, peri-implantitis and healthy controls. In contrast to earlier studies, the healthy controls were periodontally healthy individuals. Their analysis identified up-regulation of genes related to bacterial response systems in patients with periodontitis compared to healthy controls. However, the study group was small, including only seven patients with periodontitis and seven healthy controls and the results should therefore be interpreted with caution.[174]

The studies described above have reported genes that are up- and down-regulated in periodontitis, as well as their related functions. However, to develop better understanding of this chronic condition, follow-up studies should be performed to elucidate the mechanisms by which these genes contribute to periodontitis. Besides that, gene expression data obtained from these studies originated from biopsies with heterogeneous cell populations, including epithelial cells, fibroblasts, and infiltrating inflammatory cells. Novel methods that can associate gene expression with different cell types, as discussed in the previous section, are important to increase the knowledge of the complex disease periodontitis.

TAXONOMIC CHARACTERIZATION OF PERIODONTITIS – PREVIOUS STUDIES

Historically, identification of bacterial taxa has relied on culture-based methods. Later on, when microarrays became started to be used for genomic analyses, they were applied also for investigations of the oral microbiome. With subsequent advancements of sequencing technologies, the understanding of the oral microbiome has been largely expanded and well studied. This section will mainly cover recently reported studies, with focus on studies using high-throughput sequencing and including a relatively high number of samples.

A study that is worth mentioning is one by Socransky et al in 1998, although this study was not recent and did not use high-throughput sequencing.[28] In this study, microbiota identified in plaque samples were grouped into five complexes associated with health or disease.[28] Since then, more taxa have been identified, some of them having as good or better correlation with periodontitis than the red complex.[25,175,176] However, the study by Socransky and colleagues was ground-breaking in the sense that it was the first study that put forward the idea that periodontitis is not initiated by a single species, but rather by a dysbiotic community of microbiota.[177]

Regarding subgingival plaque samples, in 2012, Griffen and co-workers reported on bacterial communities from 29 patients with periodontitis and 29 healthy controls.[176] In this study, the phyla *Spirochaetes*, *Synergistetes*, and *Bacteroidetes* were more abundant in samples from patients with periodontitis, whereas *Proteobacteria* was more abundant in health. Species reported as periodontitis-associated included *Filifactor alocis*, *P. gingivalis* and *T. denticola*, whereas *Streptococcus mitis*, *Streptococcus sanguinis* and *Moraxella osloensis* were health-

associated.[176] Besides that, Abusleme et al investigated the microbial communities in subgingival plaque samples from 22 patients with periodontitis and 10 healthy controls.[175] Plaque samples from patients with periodontitis demonstrated higher proportions of *Spirochaetes*, *Synergistetes*, *Firmicutes* and *Chloroflexi*, whereas *Actinobacteria* was more abundant in health.[175]

In 2013, subgingival plaque samples from 88 patients with periodontitis were investigated by Ge *et al*.[178] Two samples were collected from each subject; one sample from a deep pocket and the other one from a shallow pocket. Phyla *Bacteroidetes* and *Spirochaetes* were more abundant in deep pockets, whereas *Firmicutes*, *Actinobacteria* and *Proteobacteria* were reduced in number. At species level *P. gingivalis*, *Porphyromonas endodontalis*, *Fusobacterium nucleatum*, *Prevotella nigrescens*, *T. denticola*, *Treponema medium*, *T. forsythia* and *Parvimonas micra* were overrepresented in deep sites, whereas species overrepresented in shallow sites included *Streptococcus oralis*, *S. sanguinis*, *Streptococcus gordonii*, *Rothia dentiocariosa*, *Veillonella dispar*, *Actinomyces naeslundii* and *Actinomyces* sp.[178]

In 2014, Li *et al.* interrogated 25 subgingival plaque samples from periodontitis patients and 13 healthy control samples.[179] At phylum level, *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, *Tenericutes*, *Chloroflexi*, *Synergistetes* and *Euryarchaeota* were periodontitis-associated, whereas *Proteobacteria* was more abundant in health. *Fusobacterium*, *Porphyromonas*, *Treponema*, *Filifactor*, *Eubacterium*, *Tannerella*, *Hallella*, *Parvimonas*, *Peptostreptococcus* and *Catonella* were increased in periodontitis. The genera *Neisseria*, *Corynebacterium*, *Capnocytophaga* and *Actinomyces* were more abundant in samples from healthy controls. In this study, *Filifactor* was also emphasized as prevalent and strongly associated with periodontitis.[179]

Next, Kirst *et al.* (2015) interrogated 25 plaque samples from patients with periodontitis and 25 from healthy controls.[180] The research group reported the species *P. gingivalis*, *T. denticola*, *T. forsythia*, *F. nucleatum*, *F. alocis* and *Fretibacterium* sp. as more abundant in periodontitis. *Actinomyces* sp., *S. mitis*, *Streptococcus sanguis*, *Gemella haemolysans*, and *Granulicatella adjacens* were associated with health.[180]

Regarding saliva samples, fewer studies have been reported on the microbial composition in periodontitis. Among reported studies few have included a large cohort and used high-throughput sequencing. One large study (139 patients with periodontitis and 447 healthy controls) was conducted by Belstrom *et al,* (2014) using microarray technique for taxonomic characterization of the salivary microbiota. Taxa that were reported as more abundant in periodontitis included *T. forsythia*, *P. micra*, and *F. alocis*.[181] Moreover, a vast study was recently reported on samples from inhabitants of a town in Japan (2,343 participants), performed using 16S rRNA gene sequencing.[182] In this study, the authors did not report on specific taxa associated with clinical periodontitis and health, but they reported that the phylogenetic diversity increased with clinical measures indicative of periodontitis, such as mean PPD, % teeth with BOP and plaque index.[182]

To summarize, the pathogenesis and etiology of periodontitis are gradually being elucidated, to a large extent spurred by technological advancements. Nonetheless, neither the precise mechanisms behind the pathogenesis of periodontitis, the interaction between microbiota and host-immune response, nor the link with systemic diseases are completely understood, and currently there are today no biomarkers with higher diagnostic precision than clinical examinations. Therefore, in this thesis, we have interrogated the transcriptome and microbiota associated with periodontitis by using massively parallel sequencing techniques in order to add to the knowledge of this complex disease, and to identify candidate biomarker proteins and microbes for future diagnostic assessment of periodontitis.

AIMS OF THE THESIS

The overall aims of this thesis were to identify the genes involved in the pathogenesis of periodontitis, and to identify candidate biomarkers by investigating the transcriptomic and taxonomic profiles in gingival tissue or saliva samples from patients with periodontitis and healthy controls.

STUDY I

To characterize the global transcriptome of periodontitis, as well as its association to other chronic inflammatory disorders, by RNA-seq of gingival biopsies from patients with periodontitis in comparison to biopsies from healthy controls.

STUDY II

To investigate the levels of MUC4 and MMP7 in saliva and GCF samples from periodontitis patients and healthy controls.

STUDY III

To characterize the taxonomic composition of the salivary microbiota in periodontitis and the association between pathogens and the host's immune and inflammatory mediators in saliva samples for periodontitis risk assessment.

STUDY IV

To develop a method that allows visualization and quantitative analysis of the transcriptome with spatial resolution in individual tissue sections.

STUDY V

To simultaneously quantify and localize gene expression in periodontitis-affected gingival tissue using the spatially resolved transcriptome analysis technique developed in *Study IV*.

MATERIALS AND METHODS

This section gives a brief overview of all the methods used to obtain the results presented in this thesis. For more detailed protocols, including in-depth details such as concentrations or sources of reagents, the reader is referred to the materials and methods sections of the published studies and manuscripts provided in the appendix.

ETHICAL CONSIDERATIONS

All of the studies presented in this thesis were performed in accordance with the Declaration of Helsinki and current Swedish legislation. Ethical permit for collection of gingival tissue biopsies (*Studies I and V*) was approved by the Regional Ethics Board in Stockholm, Sweden. Collection of oral cells (*Study I*) was approved by the Regional Ethics Board in Stockholm. Collections of saliva and GCF samples (*Studies II and III*) were approved by the Regional Ethics Board in Stockholm or by the Ethical Review Board at the University of Lund, Sweden. Ethical permits for the collection of mouse olfactory bulbs and breast cancer biopsies, included in *Study IV*, were approved by the Stockholm North Animal Ethics Committee and the Ethical Review Board at the University of Lund, respectively. In all studies that involved human subjects, a written informed consent was obtained from each individual participant.

SUBJECTS AND COLLECTION OF SAMPLES

Gingival tissues

A total of 144 gingival biopsies were collected for transcriptome analysis in *Study I*. The inclusion criteria for the patients with periodontitis were tooth sites with PPD ≥ 6 mm, CAL ≥ 5 mm, and BOP. The inclusion criteria for the healthy controls were PPD ≤ 3 mm, CAL ≤ 3.5 mm and no BOP. The biopsies were collected by calibrated dentists.

The gingival tissues were split in halves, where one half was stored in RNA Later solution before being subjected to RNA-seq. The other half was fixed in buffered formalin before being sectioned and stained with hematoxylin and eosin (H&E) and/or analysed with immunohistochemistry. Biopsies stained with H&E were used for assessment of degree of inflammation, as evaluated by three "blinded" observers giving a score between 0 and 3 (0 = no evidence of inflammatory infiltration, 1 = slight inflammatory infiltration, 2 = moderate inflammatory infiltration, and 3 = severe inflammatory infiltration).

For *Study V*, gingival tissues from patients with periodontitis were collected and immediately snap frozen in liquid nitrogen. The biopsies were cryosectioned onto glass slides, stained with H&E and visually inspected. The biopsy that showed the most intact histology was selected for analysis with spatial transcriptomics.

Saliva samples

Saliva samples, analysed in *Studies II and III*, were collected from two independent cohorts. In both studies, stimulated saliva samples were collected into test tubes while the subjects were chewing paraffin tablets. The samples were thereafter immediately frozen at -20 °C. The samples were thawed and centrifuged, and the supernatants were collected and aliquoted. The aliquots were stored at -80 °C until analysis.

In *Study II*, the inclusion criteria for patients with periodontitis were BOP in > 30% of sites and loss of supporting tissues exceeding 1/3 of the root length. 84 % of the patients had PPD ≥ 6 mm and 16 % had PPD 4-5 mm. The inclusion criteria for healthy controls were no signs of bone loss, BOP < 30 % and PPD < 4 mm. In total 37 individuals with periodontitis and 39 healthy controls were included in this study.

In *Study III*, 48 patients with periodontitis and 48 healthy controls were included. The inclusion criteria for patients with periodontitis were tooth sites with BOP, PPD ≥ 6 mm and CAL ≥ 5 mm. Healthy control individuals had no signs of gingival inflammation and PPD ≤ 3 mm.

GCF samples

GCF samples from 20 patients with periodontitis and 20 healthy controls were included in *Study II*. These samples were collected by inserting paper strips into the gingival crevice and thereafter immersing the strips in phosphate buffered saline containing Tween. Samples were collected from sites with PPD ≥ 6 mm from patients with periodontitis, and from sites with PPD ≤ 3 mm from healthy controls.

Cell cultures

In *Study I*, the expression and regulation of *MUC4*, *MMP7*, and pleckstrin (*PLEK*) were investigated *in vitro* in oral cells. For *MUC4* expression, studies were performed on human primary epithelial cells. As for *MMP7* and *PLEK,* experiments were performed on fibroblast cells established from gingival tissue obtained from individuals with no clinical signs of periodontitis.

TRANSCRIPTOMIC AND TAXONOMIC ANALYSES

Transcriptomic and taxonomic analyses were performed in four of the studies (S*tudies I, III, IV, and V*) included in this thesis, using massively parallel sequencing. In addition, RT-qPCR was also used in *Study I* for transcriptomic analysis. RT-qPCR is a low-throughput method suitable for analysis of a smaller number of transcripts. Therefore, in *Study I*, we used RT-qPCR for validation of three up-regulated genes.

The preparation of libraries (collections of DNA molecules ready for sequencing), for each study will be briefly covered in the sections below. In *Study I*, a library preparation protocol for RNA-seq was followed as provided by Illumina. In *Study III*, taxonomic profiling was performed by sequencing a region of the 16S rRNA gene in microbial DNA isolated from saliva samples. In *Study IV*, a novel RNA-seq method for transcriptome profiling with spatial resolution was developed, with an in-house developed library preparation protocol, which also was applied to a gingival biopsy in *Study V*.

RT-qPCR

In *Study I*, RT-qPCR was employed to analyse the mRNA expression of *MUC4*, *MMP7*, and *PLEK* after stimulation with LPS in oral cells. The extracted RNA was first reverse transcribed into complementary cDNA and thereafter qPCR was performed using the cDNA as template.

The TaqMan method was employed, which uses sequence specific reporter probes.[183] The reporter probes, which contain both fluorophore and quencher anneals to the DNA template. When the polymerase synthesizes a new copy of the DNA during PCR, it degrades the reporter probes upon encountering, which separates the fluorophore and the quencher. This separation of the fluorophore and quencher creates a fluorescent signal that can be measured.[183] In *Study I*, primers and reporter probes specific for *MUC4*, *MMP7*, *PLEK*, and, as reference, the housekeeping gene glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*) were used.

Massively parallel sequencing

In *Studies I, III, IV, and V,* massively parallel sequencing was mainly employed. In these studies we used the Illumina sequencing platform, which today is the largest sequencing technology on the market. In this section, library preparation and sequencing are described as below for *Studies I and III*, while alterations to these protocols, as incorporated in *Studies IV and V*, are covered in the next section.

In *Study I*, RNA was first extracted from the 144 biopsies and the RNA Integrity Number (RIN)[184] was measured. Samples with RIN < 7 and yield < 0.4 μg were excluded from further analyses, leaving 65 samples from patients with periodontitis and 64 from healthy controls. Thereafter, libraries were prepared with the Illumina stranded TruSeq protocol, which includes capture of polyA-RNA using polyT-coated magnetic beads, fragmentation of the RNA, reverse transcription, second strand synthesis with dUTP incorporation, sequencing adapter ligation, and amplification of adapter-ligated fragments by PCR, according to instructions provided by Illumina. Before proceeding to sequencing, a size selection step was performed to eliminate free adapters and primers.

For *Study III*, DNA was extracted from all 96 saliva samples and a region of the 16S rRNA gene was amplified using PCR. After a clean-up step to remove free primers and potential primer-dimers, a second PCR step was performed in which the sequencing adapters were incorporated followed by one additional clean-up step, before proceeding to sequencing.

Spatially resolved sequencing

In *Study IV*, a new technique for spatially resolved RNA-seq was developed, and this method was also used for interrogation of gene expression within a periodontitis-affected gingival tissue section in *Study V*.

In summary, this method utilizes a glass slide with primers printed onto it in an array-format. Each spot on the array contains primers with base pair sequences unique for each spot

(hereafter called barcodes), and polyT-sequences. A tissue section is placed onto the array and then stained with H&E and imaged under a microscope, in order to identify histological structures to be associated with the gene expression data. RNA is allowed to diffuse out of the tissue section and hybridize to the polyT-sequences of the primers on the array. Reverse transcription is thereafter performed *in situ*, resulting in barcoded cDNA attached to the glass surface. Thereafter, the tissue is removed from the glass slide and discarded while the barcoded cDNA is enzymatically cleaved off the glass slide and collected. To prepare libraries for sequencing, the collected cDNA is first subjected to second strand synthesis. Next, *in vitro* transcription is performed, which produces linearly amplified RNA fragments. The *in vitro* transcription is followed by ligation of sequencing adapters and another round of reverse transcription to produce cDNA. Finally, the fragments are amplified by PCR prior to sequencing. Each of the above-mentioned steps is followed by a clean-up step.

ANALYSIS OF SEQUENCING DATA

A sequencing run typically generates millions of raw data reads. The sequencing data are usually reported from the sequencer as FASTQ files, containing the actual sequences of bases read by the instrument, together with the quality of each base in the sequencing reads. These files need to be processed into interpretable information. The first step is to convert the sequences into gene or microbial counts. Thereafter, biological interpretations need to be made from the feature counts data, often by comparing two conditions, such as a pathological disease state against normal healthy.

Quantification of transcripts or microbes

All the presented studies in this thesis used paired end sequencing, which means that the sequencer reads each fragment from both ends and therefore reports two sequences for each fragment. In *Study I*, both reads in each read pair were aligned to a human reference genome. Duplication rates were calculated and samples having a high number of duplicated reads were deemed as having low quality and were excluded from further analysis, leaving 62 out of 65 samples from patients with periodontitis and 62 out of 64 samples from healthy controls. Thereafter, the alignments were annotated to genes and quantified. Only pairs of reads where both reads were annotated to the same gene were included for analysis.

In *Study III*, three samples which had less than 10,000 reads were excluded from further analysis, resulting in 46 samples from patients with periodontitis and 47 from healthy controls to be included in the analysis. The sequencing reads were processed with the Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline, which uses an error model, based on the quality scores provided by the sequencing platform, to correct reads.[185] The corrected read pairs were merged and aligned to the Ribosomal Database Project (RDP) database and annotated at Phylum, Class, Order, Family, Genus and Species levels.

In *Studies IV and V*, from each generated pair of reads, one read contained the transcript and the other read contained the barcode. The reads containing the transcripts were aligned and annotated to a reference genome. The other read, containing the sequence from the barcode, was mapped against a reference file for translation into corresponding *x* and *y* coordinates on the array.

Biological interpretation of transcripts/microbe abundances

In *Study I*, exploratory data analysis was first performed using the multivariate statistical model Principal Component Analysis (PCA). Multivariate modelling is used to reduce dimensionality of complex data to render it easier to be explored and visualized. PCA is a commonly used dimensionality reduction method that transforms data into linear components, which can for example be visualized in a scatterplot.

In addition to PCA, another dimensionality reduction technique, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA; alternatively Orthogonal Projections to Latent Structures Discriminant Analysis) was used in *Study I*. OPLS-DA also calculates linear components, but also takes the outcome into consideration, which in this case is the disease status (periodontitis/healthy) and tries to find the variance within the dataset that best separate these two groups. Furthermore, variables with high reliability that influence the grouping separation (periodontitis/healthy) in the OPLS-DA can be identified, by constructing an S-plot.[186]

Significant Analysis of Microarray Sequencing (SAMSeq), which uses both non-parametric testing and a resampling strategy to equalize library sizes, was employed to identify the dysregulated gene expression that differed between periodontitis and healthy groups.[187] The reported up- and down-regulated genes were analysed for Gene Ontology (GO) categories in order to find functions that differed between periodontitis and health. Besides that, the lists of up- and down-regulated genes were also compared to differentially expressed genes downloaded from previous studies on systemic inflammatory conditions. A literature search for transcriptome analyses of diseases previously implied as associated with periodontitis was conducted. Available studies performed on biopsies from patients with disease and healthy controls, which reported up- and down-regulated genes, or raw data, were found for CVD, RA or UC. Differentially expressed genes were downloaded from these studies, or, in case of CVD, raw data was downloaded and processed as described by the authors of the study.[188-192]

In *Study III*, the microbial data was first analysed by Principal Coordinate Analysis (PCoA), which is similar to PCA but is based on a distance matrix. In addition, sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was used to identify differences between periodontitis and healthy based on both microbial data and levels of inflammatory mediators. This classification method is similar to OPLS-DA, however it contains a selection scheme where only a subset of the variables (microbiota and levels of inflammatory mediators) relevant for separating the classes is retained.[193]

For identifying microbiota that differed significantly between saliva samples from patients with periodontitis and healthy controls, DESeq2 was used, which includes median ratio normalization and negative binomial model of the count data that forms the basis of the statistical test.[194] In addition, the machine learning method random forest was used as a second approach for identification of microbiota whose abundances differed between periodontitis and health.[195] Random forest is a machine learning method used for classification of groups (in this case periodontitis and health), but it is also possible to extract variables important for the classification.[195] In *Study III* this method was exploited for identifying microbiota specific for periodontitis and health.

In *Study V*, the data was normalized by the median of ratios approach implemented in DESeq2. To visualize the high-dimensional data in a lower dimensional space, t-distributed Stochastic Neighbour Embedding (t-SNE) was used, which is a non-linear method that projects high-dimensional data in a two-dimensional scatter plot so that samples similar to one another clusters together and dissimilar points are placed more far apart from each other.[196]

For investigating the localization of genes within subareas in the tissue section, the gene expression data was plotted in a scatter plot according to the *x* and *y* coordinates of each spot and with the spots coloured according to gene expression levels. This generated scatter plot was thereafter overlaid with the earlier captured image of the H&E stained tissue section. For identifying genes differentially expressed between different subareas, spots were selected based on where the spots were located, either within inflamed or non-inflamed regions. DESeq2 was used for identifying differentially expressed genes between these subareas.

PROTEIN EXPRESSION ANALYSES

For investigation of protein levels in gingival tissue as well as in saliva and GCF samples, the following techniques immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and Bio-plex multiplex immunoassay were employed in *Studies I, II, and III*. These techniques are similar in the aspect that they are based on antibody detection, but differ in the type of specimen they can analyse, as well as their throughput. Immunohistochemistry is employed for expression analysis of tissues and cells, whereas both ELISA and Bio-Plex are used for analysis of liquid samples.

Immunohistochemistry

Immunohistochemistry was employed to study the degree of inflammation within the gingival tissue, and the proteins expression levels corresponding to the genes identified by RNA-seq (*Study I*).

To investigate B-cells and macrophages, tissues were stained with antibodies against cluster of differentiation 20 and 68 (CD20 and CD68), respectively. To confirm findings from the RNA-seq analysis, gingival tissues were stained with antibodies against MUC4, MMP7, and

PLEK. Immunohistochemical analyses were performed using primary monoclonal antibodies directed against CD20, CD68, MUC4, MMP7, or PLEK. After incubation with the primary antibodies, horseradish-peroxidase (HRP)-conjugated secondary anti-mouse antibodies were added to the slides, followed by addition of diaminobenzidine, which was oxidized by HRP to form a coloured product in the areas of the protein of interest. Finally, the slides were mounted and observed under a light microscope.

The stainings were evaluated by three "blinded" observers. In case of MMP7, the extent of stained cells in the epithelium and connective tissue were assessed using a relative scale from 0 to 3. The sections stained for MUC4, PLEK, CD20, or CD68 were examined for the presence or absence of stained cells in the epithelium and/or connective tissue.

ELISA

Pre-fabricated ELISA kits were employed to analyse the protein levels of MUC4 and MMP7 in saliva and GCF samples in *Study II*. Briefly, the samples were added to wells pre-coated with capture antibodies directed against MUC4 or MMP7. Detection antibodies specific for MUC4 or MMP7 were added to the wells followed by addition of enzyme-linked secondary antibodies. Thereafter, a substrate was added, which was converted by the enzyme into a yellow coloured product. Finally, the absorbance was measured by a spectrophotometer to determine the concentration of MUC4 or MMP7 within the samples.

Bio-Plex multiplex assay

Bio-Plex was utilized in *Study III* to measure the levels of inflammatory mediators in saliva samples. Bio-Plex is similar to ELISA, however, with the capturing antibodies coated onto small beads (micrometer scale) instead of on the bottom of wells. In addition, the secondary antibodies are linked to biotin, which is detected by addition of streptavidin-coupled fluorophores, to increase the specificity and sensitivity of the assay.

Multiple analytes can be investigated simultaneously in the same sample by including beads coated with antibodies against different analytes. To allow differentiation of beads, they are internally dyed with red and infrared fluorophores of different intensities where a specific known ratio of the two dyes are associated with a specific antibody. During analysis, the beads are read one by one and the combination of dyes (corresponding to a specific analyte) and fluorophore strength, reflecting the analyte's concentrations, are detected and read.

STATISTICAL ANALYSES

In this section, statistical analyses other than those used for sequencing will be covered. This includes analysis from protein expression interrogations, RT-qPCR, and comparisons of patient characteristics.

In *Study I*, the degree of inflammation was evaluated by three observers. For each sample, the median value for the three observers was calculated. The mean of the medians was obtained

from each group (periodontitis and healthy) and the difference between samples from patients with periodontitis and healthy controls was assessed using Mann-Whitney U-test. The amount of MMP7-positive cells in the epithelium and connective tissue was analysed similarly as described above. For MUC4, CD20, and CD68, the sections were examined for the presence of specific-stained cells. The values for the biopsies from periodontitis patients and healthy controls were compared using Fisher's exact test.

The results from the RT-qPCR analysis in *Study I* were calculated with the ΔΔCt method[197] where the mean Ct for *MUC4*, *MMP7*, and *PLEK* were normalized against the mean Ct for *GAPDH* for each sample. All cell-based experiments were performed using cells from at least three individuals and each experiment was run with three technical replicates. The significances of the differences between cells stimulated with LPS and unstimulated control cells were assessed by Students *t*-test (two-tailed).

In *Study II*, comparisons of patient characteristics were performed with Mann-Whitney U test for continuous variables. For dichotomous variables, cross tables were constructed and in case all cell values exceeded five, Chi-square χ^2 test was used, otherwise Fisher's exact test was used. Experimental results in *Study II* were evaluated by univariate analyses and regression models. The significance of difference of MUC4 and MMP7 levels between samples from patients with periodontitis and healthy controls were assessed by Mann-Whitney U-test. Multiple regression analyses were performed with MUC4 or MMP7 levels as dependent variables, after log-transformation to achieve normality. Periodontitis status, age, and smoking habits were included as independent variables. In addition, logistic regression was performed with periodontitis as dependent variable and the ratio of MUC4/MMP7 related to total protein concentrations, as well as age and smoking habits, was included as independent variables.

In *Study III*, significance of differences between levels of inflammatory mediators in saliva samples from patients with periodontitis and healthy controls were assessed with Mann-Whitney U-test. The *p*-values were adjusted with Benjamini-Hochberg post-hoc test.

For all studies described above, *p*-values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The studies in this thesis focus on transcriptomic and taxonomic characterization of periodontitis. The transcriptome profiles of gingival tissue biopsies from patients with periodontitis were interrogated in *Study I*. In *Study II*, the proteins translated from the genes that were most highly associated with periodontitis, as identified in *Study I*, were analysed in saliva and GCF samples of periodontitis patients and healthy controls. Saliva samples were also investigated in *Study III*, with focuses on the microbiome of saliva in periodontitis and health. In *Studies IV and V*, the focus was again on transcriptome analysis, where, in *Study IV*, a technique for transcriptome analysis within tissue sections was developed, which was then applied to a gingival tissue biopsy from a patient with periodontitis in *Study V*. This section gives a brief overview of the results from these studies and a discussion of the findings related to current literature.

Degree of inflammation in gingival tissue biopsies from patients with periodontitis and healthy controls (*Study I*)

In *Study I*, gingival biopsies were collected from patients with periodontitis and healthy controls. Each collected biopsy was divided in half where one half was subjected to RNA-seq, and the other half was fixed in buffered-formalin for histochemical analysis.

Infiltration of inflammatory cells is one of the hallmarks of periodontal disease and therefore we initially investigated the extent of infiltration of inflammatory cells in gingival tissue of patients with periodontitis and healthy individuals. We assessed the degrees of inflammation of all biopsies by H&E staining as well as with cell-type-specific antibodies in 20 biopsies from periodontitis patients and 20 from healthy controls. Assessment of the H&E staining revealed significantly ($p < 0.01$) more infiltrating inflammatory cells in the periodontitis group. The biopsies from the patients with periodontitis also had significantly higher expressions of the B-cell marker CD20 and the macrophage marker CD68 ($p < 0.01$, and $p = 0.02$, respectively).

Gene expression patterns related to periodontitis and health (*Study I*)

The gene expression data was first investigated with multivariate methods in order to explore the overall patterns of gene expression of all samples, using PCA. In the PCA plot, the samples were labelled with colours either according to their degree of inflammation (as determined by histochemical analysis), or according to their disease status (periodontitis/healthy). This analysis revealed that the largest variation of gene expression within all samples was related to the degree of inflammation. The second largest variation was related to the disease status of the participant. The fact that the PCA identified gene expressions that differed between biopsies from patients with periodontitis and healthy individuals irrespective of the degree of inflammation, indicates that other mechanisms besides inflammation are involved in the transition from health to periodontitis.

In addition to multivariate analysis, univariate differential expression analysis was performed on the gene expression data. Our statistical analysis revealed that 665 genes were up-regulated in periodontitis and 633 genes were down-regulated in periodontitis. The significantly up- and down-regulated genes were further analysed for enriched GO categories. The GO analysis demonstrated that the up-regulated genes were most strongly associated with functions relating to inflammation, defence, and wounding responses, as well as functions associated with apoptosis and cell death. On the other hand, down-regulated genes were related to organization of extracellular matrix, extracellular structure and collagen fibrils, development of skeletal system, bone, skin, ectoderm, and ectodermis, as well as ossification and osteoblast differentiation. Thus, the up-regulation of inflammatory processes in periodontitis is consistent with our initial findings that the degree of inflammation was higher in biopsies from periodontitis patients compared to healthy controls. In addition, the up-regulation of apoptotic processes and the down-regulation of processes related to structural support and development of skin and bone are consistent with clinical manifestations of periodontitis, which include loss of structural tissue and bone.

Subsequent analysis of the differentially expressed genes revealed *MUC4* and *MMP7* as the two most highly up-regulated genes in periodontitis. To our knowledge, neither *MUC4* nor *MMP7* has previously been reported as highly associated with periodontitis. The protein MUC4 is a member of the mucin family of glycoproteins, which up to date has 20 members.[148] Mucins are produced by mucus-producing epithelial cells after stimulation by microbial products and inflammatory mediators, including TNF-α and IL-1β, and by PMNs via neutrophil elastase.[199-206] Although MUC4 has not previously been implicated in periodontitis, it has been reported as involved in cancer processes by mediating the epidermal growth factor family of enzymes, including the tyrosine kinase receptor ERBB2. This results in activation of downstream signal transduction pathways, including protein kinase C (PKC), which is involved in inflammation.[207-209]

The enzyme MMP7 belongs to the MMP family of enzymes, which are the main proteins responsible for remodeling of the extracellular matrix; they are able to degrade basement membrane and extracellular matrix components, and contribute to the pathogenesis of periodontitis through destruction of periodontal tissue.[124,210,211] In contrast to MMP7, other MMPs, mainly MMP2, 8, and 9, have previously been associated with periodontitis.[124,212,213] Notably, our study is the first to report an association between MMP7 and periodontitis, highlighting this MMP as potentially important for the pathogenesis of periodontitis.

Expression of *MUC4*/MUC4 in gingival tissue, oral cells, saliva, and GCF (*Studies I and II*)

Our finding that *MUC4* was one of the two most highly up-regulated gene in periodontitis prompted us to further investigate the expression of the protein MUC4 in gingival tissue, the expression and regulation of *MUC4* in oral cells, as well as the protein levels of MUC4 in saliva and GCF samples. First, the expression of *MUC4* was verified at protein level in gingival tissue from patients with periodontitis and healthy controls. Immunohistochemical staining of 20 gingival biopsies from patients with periodontitis and 20 from healthy controls confirmed over-expression of the protein MUC4 in gingival tissue. MUC4 was detected in the gingival epithelium of 18 (90%) patients with periodontitis but only in 2 (10%) of healthy controls ($p < 0.001$).

The regulation of *MUC4* was thereafter investigated *in vitro* in oral cells. Due to the fact that the immunohistochemical analysis revealed MUC4 to be expressed only in the epithelium, the expression and regulation of *MUC4* were analysed in epithelial cells. The cells were stimulated with LPS for 24 hours, which resulted in a significant ($p < 0.01$) increase of *MUC4* expression, in comparison with cells treated with culture medium only.

In *Study II*, the levels of MUC4 were further studied in saliva and GCF samples from patients with periodontitis and healthy individuals, with the purpose to explore the potential of MUC4 as a diagnostic marker for periodontitis. The analysis revealed that the levels of MUC4 were significantly decreased both in saliva and GCF samples ($p < 0.01$ and $p < 0.05$, respectively) of patients with periodontitis compared to controls. The association between periodontitis and

levels of MUC4 remained significant ($p < 0.05$) even after adjusting for total protein concentrations, age, and smoking, both regarding saliva and GCF samples.

The contrasting findings of MUC4 expression levels in gingival tissue compared to saliva and GCF may be due to the fact that MUC4 can be both in secreted and membrane-bound forms.[199,214-216] The balance between these forms might be altered in response to inflammation.[217] With regard to previous studies on mucins in saliva, it has previously been reported that levels of mucins are increased in saliva samples of periodontitis patients compared to healthy controls. However, these studies reported on mucins in general and did not distinguish between the different mucin family members.[218-220]

Expression of *MMP7*/MMP7 in gingival tissue, oral cells, saliva, and GCF (*Studies I and II*)

The second most highly up-regulated gene in gingival tissue from patients with periodontitis compared to healthy controls was *MMP7*. The expression of the protein product of the *MMP7* gene was also verified in gingival biopsies by immunohistochemical staining. Evaluation of the MMP7 protein expression in gingival tissue revealed a significantly ($p < 0.01$) elevated expression of MMP7 in connective tissue of gingival biopsies from periodontitis patients compared to controls (mean score 2.1 and 1.0, respectively). In contrast, there were no significant differences in expression of MMP7 in the epithelium of gingival tissues ($p = 0.40$). Our results that showed that MMP7 is overexpressed in connective tissue of gingival tissue of patients with periodontitis, but not in the epithelium, supports the suggestion that MMP7 is continuously expressed in epithelial cells to provide protection against microorganisms.[221]

The regulation of *MMP7* was also studied in *in vitro* experiments. Based on our findings that MMP7 was differentially expressed in the connective tissue and not in the epithelium, we used gingival fibroblasts, which are the most ubiquitous cells of the gingival connective tissue, for these experiments. Treatment of these cells with LPS for 24 hours significantly ($p < 0.001$) increased the gene expression of *MMP7* in gingival fibroblasts.

In addition, the protein expression of MMP7 was also determined in saliva and GCF samples from periodontitis patients and healthy controls (*Study II*). The MMP7 levels were significantly elevated in saliva and GCF samples ($p < 0.05$ and $p < 0.01$, respectively) of periodontitis patients compared to healthy individuals. However, the levels of MMP7 were not significantly increased in periodontitis after adjusting for total protein concentrations, age, and smoking. Levels of MMP7 have, to our knowledge, not previously been reported as an enriched marker in saliva samples from periodontitis patients as compared to healthy controls. Other members of the MMP family has however previously been reported as up-regulated in saliva during periodontitis, most notably MMP8.[119,120,123,222] MMP7 can activate MMP8, thereby potentially promoting maintenance of periodontitis.[223]

Up-regulation of *PLEK* in periodontitis, CVD, RA, and UC (*Study I*)

To identify common genes that might act as links between periodontitis and other chronic inflammatory diseases, we compared the genes differentially expressed in periodontitis (obtained from *Study I*) with genes up- and down-regulated in CVD, RA, and UC (obtained from previous studies). Our analysis revealed that similar processes, related to immune responses, cell motion, cell death, and homeostasis were up-regulated in all four diseases. The comparison revealed only one gene commonly up-regulated in all four diseases – *PLEK*. To further investigate our novel finding that *PLEK* was commonly up-regulated among the diseases investigated here, *PLEK* mRNA expression and regulation was analysed in oral cells and the protein expression was analysed in gingival tissue.

Expression and regulation of *PLEK* was studied *in vitro* using gingival fibroblasts and epithelial cells. *PLEK* mRNA expression was significantly ($p < 0.01$) up-regulated in gingival fibroblasts in response to stimulation with LPS. In contrast, the mRNA expression of *PLEK* in epithelial cells was unaffected by LPS treatment. The protein expression of PLEK was also verified in biopsies of periodontitis patients and healthy individuals. PLEK was detected, by immunohistochemical staining, in association with fibroblast-like cells, epithelial cells, immune cells, and endothelial cells of gingival tissue from patients with periodontitis. In contrast, immunohistochemical analysis of gingival biopsies from healthy controls showed low proportion of stained cells.

PLEK is an inducible PKC substrate expressed by macrophages implied to play an important role in the secretion and activation pathways of the pro-inflammatory cytokines TNF-α and IL-1β.[224,225] In addition, it has been reported that PLEK is up-regulated in macrophages after stimulation with LPS, which is in line with our findings that PLEK is up-regulated in gingival fibroblasts after stimulation with LPS.[226]

The reason for identification of only one common gene may depend on the fact that we compared data from our RNA-seq study with data obtained using microarrays. RNA-seq has been shown to outperform microarray technology, mainly due to higher precision in detecting transcript levels, which allows for better identification of differentially expressed genes.[141,144-147] However, additional studies are required to explore the involvement of PLEK in inflammatory diseases.

Salivary microbial signatures in periodontitis (*Study III*)

Periodontitis is induced by accumulation of microbes residing close to the gingiva, which triggers a host-immune response. The main objective of *Study III* was therefore to characterize the taxonomic composition of saliva samples from patients with periodontitis and healthy controls, by sequencing of the 16S rRNA gene. First, multivariate analysis was performed, which identified patterns of microbial compositions that differed significantly ($p < 0.001$) between saliva samples from patients with periodontitis and healthy individuals. The most abundant phyla across all samples were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. However, *Actinobacteria*, *Spirochaetes*, *Synergistetes*, *Tenericutes,* candidate

division SR1 and *Candidatus Saccharibacteria* were significantly (adjusted $p < 0.05$) differentially abundant between periodontitis and health. *Actinobacteria*, *Spirochaetes*, *Synergistetes*, and *Tenericutes* were increased in saliva samples from patients with periodontitis, whereas candidate division SR1 and *Candidatus Saccharibacteria* were enriched in samples from healthy controls.

In comparison with studies performed on plaque samples, our results from saliva samples are largely consistent with previous findings. Our findings that the phyla *Spirochaetes*, *Synergistetes*, and *Tenericutes* were increased in samples from patients with periodontitis are in agreement with previously published results.[175,176,178,179] SR1, which we identified as more abundant in saliva samples from healthy controls, has previously been reported at low levels in saliva from healthy individuals, but it has not been reported as enriched in health compared to periodontitis.[23] Nonetheless, our findings regarding *Candidatus Saccharibacteria* and *Actinobacteria* are in contrast to other published results.[179,227] This discrepancy can be explained by differences in bacterial composition between plaque and saliva samples or technical variations between studies. For example, the results reported by Liu *et al.* (2012) identifying higher abundance of *Candidatus Saccharibacteria* in periodontitis included only a few plaque samples, from two patients with periodontitis and three healthy controls, and may not be generalizable for a larger study population.[227]

At microbial level, bacterial species that had increased abundance in saliva samples from periodontitis patients compared to healthy controls included *Prevotella* sp., *Phocaeicola* sp., *Fretibacterium* sp., *Streptococcus mitis/parasanguinis*, *Eubacterium saphenum*, *T. forsythia*, *F. alocis*, and *P. micra*. Among these, four microbes could be assigned at species level, i.e. *E. saphenum*, *T. forsythia*, *F. alocis*, and *P. micra*; microbes that all have previously been associated with periodontitis in plaque samples.[176,228-233] Moreover, *F. alocis* has also been reported as increased in saliva samples from patients with periodontitis compared to healthy controls.[181] Interestingly, this microbe has also been proposed as the centre of a group of pathogens that are associated with periodontitis.[229] Regarding the microbe that was assigned to both *S. mitis* and *S. parasanguinis* (meaning that these bacteria share the same sequence for the 16S rRNA gene region), has in case of *S. mitis* previously been reported as one of the early colonizers of the periodontal biofilm, whereas *S. parasanguinis* has been associated with aggressive periodontitis.[28,234] The microbes that could not be assigned to specific species included microbes of the genera *Prevotella*, *Phocaeicola*, and *Fretibacterium*. Species belonging to the *Prevotella* genus, and most frequently *P. intermedia*, have previously been reported as associated with periodontitis.[235,236] Interestingly, *Fretibacterium* species are gaining attention for their association with periodontitis.[225,229] It is also worth to mention that *Phocaeicola* species has previously been reported as more abundant in periodontitis by one study.[233,237,238]

Salivary levels of inflammatory mediators (*Study III*)

In addition to studying the salivary microbial composition, in *Study III*, the levels of 37 different inflammatory mediators were analysed in the same saliva samples by Bio-Plex

immunoassay technology. The analysis revealed that glycoprotein 130 (gp130), soluble TNF-receptor 1 (sTNF-R1), and IL-19 were significantly ($p < 0.05$) enriched in saliva samples from periodontitis patients compared to controls. In contrast, the anti-inflammatory cytokine IL-10 was significantly ($p < 0.05$) increased in samples from healthy individuals. However, after adjusting for multiple testing, only the levels of gp130 remained significantly (adjusted $p < 0.05$) different when comparing the periodontitis patients with controls.

The gp130 co-receptor subunit (also known as soluble IL-6 receptor β [sIL-6Rβ]) is required for signalling of a group of cytokines, namely IL-6, IL-11, IL-27, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), and neuropoietin (NP). These cytokines are involved in a range of biological processes, including immune response, inflammation and bone homeostasis.[239,240] Gp130 has not previously been reported as up-regulated in periodontitis. However, cytokines that signal through the gp130 subunit, namely IL-6 and OSM, have been reported as involved in periodontitis.[240-246]

Associations between microbiota and inflammatory mediators in saliva samples (*Study III*)

To investigate possible interactions between microbiota and host inflammatory response, the levels of inflammatory mediators were analysed in the same saliva samples as for the microbial composition analysis. The results showed that the inflammatory mediators gp130, sTNF-R1, sIL-6Rα, pentraxin 3, sTNF-R2, and chitinase 3-like 1 correlated positively with the microbes *Streptococcus* sp., *Selenomonas* sp., *Treponema* spp*., Selenomonas sputigena* and an unclassified *Bacteria*. The anti-inflammatory cytokine IL-10 was identified as negatively correlated to microbes assigned to *S. mitis/parasanguinis*, *Eubacterium nodatum,* and *F. alocis*, as well as to two microbes assigned to the order *Clostridiales*. In addition, IL-10 also correlated positively with *Granulicatella elegans,* a bacterium found in normal human flora.[247] These correlations may play important roles for the development and maintenance of the chronic inflammatory disease periodontitis.

Candidate biomarkers for periodontitis (*Studies II and III*)

In *Study II*, MUC4 and MMP7 were investigated as potential biomarkers for periodontitis using saliva and GCF samples. The results demonstrated that levels of both MUC4 and MMP7 could discriminate saliva and GCF samples of patients with periodontitis from samples of healthy controls. Interestingly, when MUC4 and MMP7 were analysed in combination, they had higher precision in discriminating periodontitis from health.

In *Study III*, the microbial signatures of saliva samples from patients with periodontitis and healthy controls were interrogated. Several microorganisms were identified as more abundant in saliva samples from patients with periodontitis as compared to healthy individuals. The microorganisms identified at species level might have the possibility to serve as biomarkers due to the fact that these bacteria can potentially be cultured and be utilized as antigens for diagnostic antibody production. Therefore, we propose that the bacteria that were identified at

species level, that is *E. saphenum*, *T. forsythia*, *F. alocis*, and *P. micra,* might be used as biomarkers for early detection of periodontitis.

Currently, only one biomarker, MMP8, is commercially available, in some European countries.[5,139] The enzyme MMP8 has also repeatedly been reported as elevated in periodontitis.[119,120,123,248] We propose that MMP8 together with the potential biomarkers we identified in *Studies II and III*, namely, MUC4, MMP7, gp130 and the microbial species *E. saphenum*, *T. forsythia*, *F. alocis*, and *P. micra* might have the possibility to form a panel of biomarkers for early prediction of periodontitis. However, to define a panel of biomarkers for prediction of periodontitis, prospective studies involving large cohorts should be performed.

Spatially resolved transcriptome analysis – method development and analysis of gingival tissue (*Studies IV and V*)

RNA-seq is commonly performed on homogenized whole biopsies. Gene expression data obtained from such analyses thus represent averages of the gene expression across all cells within the biopsies, and the information of the transcripts original localizations in the tissue are lost. Therefore, in *Study IV*, a novel strategy for spatially resolved analysis in tissue sections was developed and proof-of-concept experiments were performed on mouse olfactory bulb tissue. A series of analyses were carried out to evaluate the performance of this novel technique. Comparison of the total gene expression from a spatial transcriptomics experiment and corresponding experiment performed in solution revealed high correlation ($r = 0.94$), indicating that the spatial transcriptomics method does not introduce bias compared to protocols performed in solution. The results from spatial transcriptomics experiments were deemed reproducible because experiments performed on tissue replicates were highly correlated ($r = 0.97$ between adjacent tissue sections). Gene expressions in histologically similar areas were also compared, revealing high correlation ($r = 0.93$). In contrast, areas of histologically dissimilar domains of the tissue section were less correlated ($r = 0.82$) and genes differentially expressed between these domains could be identified. To investigate gene expression patterns within tissue sections, the multivariate analysis method t-SNE was performed on the gene expression data. The analysis revealed distinct gene expression signatures that could be attributed to histologically dissimilar areas within the tissue section. These analyses demonstrate that the spatial transcriptomics method can be used for simultaneous analysis of gene expression in multiple domains within a tissue section, while also enabling the visualization of gene expression distribution within the section.

In *Study V*, the spatial transcriptomics method was applied to periodontitis-affected gingival tissue to explore gene expression signatures of different cells present within gingival tissue during periodontitis. First, an overview of the gene expression data was obtained by t-SNE analysis. The t-SNE plot revealed that the gene expression within the tissue section could be clustered into three distinct groups. These groups were identified as corresponding to three histologically different areas within the tissue section; the epithelium, an inflamed region of the connective tissue, and non-inflamed connective tissue. In order to investigate gene

expression patterns differing between inflamed and non-inflamed regions within the tissue section, ten spots corresponding to inflamed connective tissue and ten spots corresponding to non-inflamed connective tissue were analysed. Differential expression analysis between these two areas was performed, which revealed 442 genes up-regulated in the inflamed area compared to the non-inflamed area. In contrast, no genes were identified as down-regulated in the inflamed area of the tissue, compared to the non-inflamed.

The three most highly up-regulated genes in the inflamed areas were *immunoglobulin lambda like polypeptide 5* (*IGLL5*), *signal sequence receptor subunit 4* (*SSR4*), and *marginal zone B and B1 cell specific protein* (*MZB1*). The genes *IGLL5* and *MZB1* have previously been reported as up-regulated in gingival tissue biopsies from patients with aggressive or chronic periodontitis, compared to healthy controls.[172,249] However, these two genes were identified from cohorts of 48 and 241 biopsies.[172,249] By spatial transcriptomics, these genes could be identified as up-regulated using only one section of a biopsy.

However, little is known about the function of the *IGLL5* gene, but it is homologous to *IGLL1*, which is involved in B-cell development.[250] The MZB1 protein regulates surface presentation and secretion of IgMs.[251-253] The second most highly up-regulated gene, *SSR4,* encodes the delta subunit of the translocon-associated protein subunit (TRAP), which is involved in translocating proteins across the endoplasmic reticulum (ER) membrane.[254-256] There are indications that *SSR4* is involved in beta cell survival in type 2 diabetes, but this gene has however, to our knowledge, not previously been reported as up-regulated in periodontitis-affected gingival tissue.[257]

In the next series of analyses, the GO categories associated with the genes up-regulated in the inflamed area were investigated. The most enriched GO terms associated with the genes up-regulated in the inflamed area of the tissue were related to functions associated with localization and targeting to the structure ER, which is responsible for the folding, processing, and trafficking of secreted and membrane-bound proteins, including many key components of the immune response.[258]

To conclude, the spatial transcriptomics method can provide new insights into gene expression of clinical samples. Here, we have applied this method to a small gingival biopsy section from a patient with periodontitis. Using this technique, combining histological analysis and RNA-seq, we could detect both genes not previously implicated in periodontitis, and genes previously reported could be confirmed in this study.

STRENGTHS AND LIMITATIONS (*STUDIES I-V*)

Study I

This study is, to the best of our knowledge, the study that has investigated the largest number of gingival biopsies from patients with periodontitis in comparison to healthy controls, using RNA-seq. Large sample sizes lead to increased statistical power to detect differentially expressed genes.[259]

The higher degree of inflammation within the biopsies from patients with periodontitis compared to controls could be interpreted either as a strength or a limitation. Infiltration of inflammatory cells is one of the hallmarks of periodontitis, and our finding that the levels of inflammatory cells were higher in tissue of periodontitis patients confirms that these patients have an active periodontal disease. On the other hand, the inflammation will result in higher expression of genes involved in inflammation that might be up-regulated also in a transient gingival inflammation, and which are thus not specific for periodontitis.

One limitation of *Study I* is the validation of two up-regulated genes only. The transcriptome and proteome do not always correlate and, because the biological functions of interest are at protein level rather than at mRNA level, it is important to validate gene expression findings at the protein level.[260] Due to lack of time and resources, this was not possible to perform in *Study I*. Nonetheless, the remaining up-regulated genes, as well as the genes down-regulated in periodontitis, deserve deeper investigation for evaluation of their roles in periodontal disease.

Study II

A limitation with *Study II* is the small sample size included in the study, and the results might therefore not be generalizable to a larger population. One advantage of this study is the validation of the findings in an additional cohort consisting of GCF samples from patients with periodontitis and healthy controls. However, the reproducibility of the findings regarding saliva needs to be investigated in larger cohorts of saliva samples.

Study III

Strengths with *Study III* include the relatively large number of saliva samples from patients with periodontitis and healthy controls, as well as the use of 16S rRNA gene sequencing. *Study III* is, to our knowledge, the study that has analysed the largest number of saliva samples from patients with periodontitis and healthy controls, using 16S rRNA gene sequencing. 16S rRNA gene sequencing can detect a wider range of taxa than hybridization based methods, which are restricted to sequences already printed onto the array. However, by sequencing only a region of the 16S rRNA gene, it is not possible to distinguish between closely related species, and some taxonomic classifications can only be made on genus level or higher.

Study IV

Spatially resolved sequencing has several advantages both compared with traditional bulk measurements and single-cell RNA-seq approaches, as described in the introduction of this thesis. A strength with the spatial transcriptomics method developed in *Study IV* is that it is a transcriptome-wide approach and no prior knowledge of the sequences of the transcripts of interest is needed. Moreover, the technique can investigate several regions within a tissue section simultaneously, and there is no need for manual picking of the regions of interest. One weakness with this technique is the limited resolution. Resolution corresponding to dimensions of single cells would give more detailed information on gene expression of specific cells within tissue sections.

Study V

Previous studies on the transcriptome in periodontitis have been performed on whole gingival biopsies. *Study V* is the first study to investigate the transcriptome within a tissue section, thus associating tissue types within the tissue section to specific gene signatures. However, the investigation of sections from only one gingival biopsy limits the statistical power and the generalizability of the findings.

MAIN FINDINGS

STUDY I

- The largest variation of gene expression within the biopsies could be attributed to the degree of inflammation, and the second largest to the disease status of the individuals (periodontitis/healthy).
- The two genes most highly associated with periodontitis were *MUC4* and *MMP7*. The protein products of *MUC4* and *MMP7* were also increased in gingival tissue biopsies from patients with periodontitis compared to controls.
- *PLEK* is commonly up-regulated in the chronic inflammatory diseases periodontitis, CVD, RA, and UC.

STUDY II

- Protein levels of MUC4 and MMP7 in saliva and GCF samples differed significantly between patients with periodontitis and healthy individuals.

STUDY III

- The microbial composition differed significantly between saliva samples from patients with periodontitis and healthy controls.
- Several microbes, including *Prevotella* sp.*, Phocaeicola* sp., *Fretibacterium* sp., *Streptococcus mitis/parasanguinis, Eubacterium saphenum*, *Tannerella forsythia*, *Filifactor alocis,* and *Parvinomas micra* were more abundant in periodontitis, compared to healthy controls.
- The inflammatory mediator gp130 was more abundant in saliva samples from periodontitis patients compared to healthy individuals.

STUDY IV

- The spatial transcriptomics method can be utilized to obtain spatially resolved gene expression profiles of tissue sections.

STUDY V

- The different compartments within gingival tissue (epithelium, inflamed and non-inflamed connective tissue) demonstrated distinct gene expression signatures.
- The genes *IGLL5*, *SSR4*, and *MZB1* were the most highly up-regulated genes in inflamed connective tissue areas compared to non-inflamed.

CONCLUDING REMARKS

In this thesis we report transcriptomic and taxonomic profiles of gingival tissue and saliva samples from patients with periodontitis and healthy individuals. Our characterization of periodontitis provides valuable insights into the pathogenesis of this common chronic inflammatory disease and suggests candidate biomarker proteins and microbes for future diagnostic assessment of periodontitis.

We have used state-of-the-art sequencing technologies to profile the gene expression and microbiota in periodontitis. *Study I* used RNA-seq to characterize the transcriptome of gingival tissue of patients with periodontitis in comparison the healthy controls. Our results revealed *MUC4* and *MMP7* as the two most highly up-regulated genes in periodontitis. In a follow-up study, we found that the protein products of these two genes differed significantly between periodontitis and health, both in saliva and GCF samples, suggesting them as potential candidate biomarkers for periodontitis (*Study II*). *Study III* investigated microbial community compositions in saliva samples using 16S rRNA gene sequencing. The analysis identified several microorganisms that were enhanced in periodontitis, suggesting them as potential biomarkers for diagnostic assessment of periodontitis.

To study gene expression with simultaneous localization of the transcripts within the tissue, a spatially resolved gene expression technique was devised in *Study IV*. This novel technique was applied to periodontitis-affected gingival tissue in *Study V*, which identified different gene signatures in the epithelium, inflamed connective tissue, and non-inflamed connective tissue, highlighting that gingival tissue is highly heterogeneous. Gene expressions in the inflamed and non-inflamed areas were compared, which identified 442 genes as up-regulated in the inflamed connective tissue. Among these, *IGLL5*, *SSR4*, and *BMZ1* were the three most highly up-regulated genes.

Study I of this thesis is to date the largest study to have interrogated the gene expression of periodontitis using RNA-seq. Moreover, spatially resolved gene expression profiling, as performed in *Study V*, has not previously been reported with regard to periodontitis. As sequencing technologies continue to improve in terms of throughput and costs, the number of studies on the transcriptome of periodontitis will probably increase. Since relatively few studies have been reported to date, this is welcome and could contribute to a more comprehensive picture of the genes that participate in this complex disease. However, to identify genes that are differentially expressed in periodontitis and health is only the first step of the process. The proteins are the molecules that are responsible for the functions within tissue, and with that in mind, the protein products of identified genes should be validated and further studied. Moreover, mechanistic and functional studies should be undertaken in order to gain further insight into the signalling mechanisms governing disease progression, which may lead to identification of therapeutic targets for the disease.

The microbial community composition associated with periodontitis has been intensively studied, mainly in subgingival plaque samples. These studies have led to increased knowledge of the etiology of periodontitis, because of the spatial proximity of plaque to infected tissue. However, saliva is more suitable for diagnostic assessment of periodontitis

than plaque, because saliva is easier to sample and can give a description of all tooth sites in the mouth. Thus, our analysis of the microbial community patterns in saliva in periodontitis and health may provide microbial biomarkers for periodontitis.

The field of periodontology has a rich history in biomarker research. However, to date, there are no commercial biomarkers available that can predict periodontitis with higher precision than clinical examinations. The expanding field of high-throughput sequencing has the potential to identify novel candidate biomarkers for periodontal disease. Future potential candidate biomarkers, as well as biomarkers that have been proposed to date, should be validated in large prospective studies. Due to the fact that periodontitis is a complex disease in which multiple factors contribute, a panel of markers will probably be more specific for diagnosis than single biomarkers. The candidate proteins and microbial biomarkers presented in this thesis may be included in such a panel.

To sum up, the studies in this thesis have employed massively parallel sequencing to characterize the gene expression and microbiota associated with periodontitis in gingival tissue and saliva samples. The results from these studies add to current knowledge of genes involved in periodontitis, and identifies candidate biomarker proteins and microbes for future diagnostic assessment of this chronic inflammatory disease.

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REFERENCES

1. Chapple IL. Time to take periodontitis seriously. BMJ. 2014;348:g2645.

2. Adler CJ, Dobney K, Weyrich LS, Kaidonis J, Walker AW, Haak W, et al. Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions. Nat Genet. 2013;45(4):450-5, 5e1.

3. Kassebaum NJ, Bernabe E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression. J Dent Res. 2014;93(11):1045-53.

4. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. Nat Rev Immunol. 2015;15(1):30-44.

5. Kinane DF, Stathopoulou PG, Papapanou PN. Periodontal diseases. Nature Reviews Disease Primers. 2017;3:nrdp201738.

6. Locker D. The burden of oral disorders in a population of older adults. Community Dent Health. 1992;9(2):109-24.

7. Sheiham A, Steele JG, Marcenes W, Tsakos G, Finch S, Walls AW. Prevalence of impacts of dental and oral disorders and their effects on eating among older people; a national survey in Great Britain. Community Dent Oral Epidemiol. 2001;29(3):195-203.

8. Slade GD. Measuring oral health and quality of life: Department of Dental Ecology, School of Dentistry, University of North Carolina; 1997.

9. Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction. Periodontol 2000. 1997;14:9-11.

10. Lindhe J, Lang NP, Karring T. Plaque-Induced Gingival Diseases. 2009. In: Clinical Periodontology and Implant Dentistry [Internet]. ProQuest Ebook Central: Wiley; [408]. Available from: https://ebookcentral-proquest-com.proxy.kib.ki.se/lib/ki/detail.action?docID=428191.

11. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet. 2005;366(9499):1809-20.

12. Yucel-Lindberg T, Bage T. Inflammatory mediators in the pathogenesis of periodontitis. Expert Rev Mol Med. 2013;15:e7.

13. Graves D. Cytokines that promote periodontal tissue destruction. J Periodontol. 2008;79(8 Suppl):1585-91.

14. Van Dyke TE. Pro-resolving mediators in the regulation of periodontal disease. Mol Aspects Med. 2017.

15. Calsina G, Ramon JM, Echeverria JJ. Effects of smoking on periodontal tissues. J Clin Periodontol. 2002;29(8):771-6.

16. Khan SA, Kong EF, Meiller TF, Jabra-Rizk MA. Periodontal Diseases: Bug Induced, Host Promoted. PLoS Pathog. 2015;11(7):e1004952.

17. American Academy of Periodontology Task Force Report on the Update to the 1999 Classification of Periodontal Diseases and Conditions. J Periodontol. 2015;86(7):835-8.

18. Buduneli N, Kinane DF. Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. J Clin Periodontol. 2011;38 Suppl 11:85-105.

19. HOMD Human Oral Microbiome Database [Available from: http://www.homd.org.

20. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol. 2000;54:49-79.

21. Guthmiller JM, Novak KF. Periodontal Diseases. In: Brogden KA GJ, editor. Polymicrobial Diseases. Washington (DC): ASM Press; 2002.

22. Gibbons RJ, van Houte J. Bacterial adherence and the formation of dental plaques. In: Beachey EH, editor. Bacterial adherence. London, United Kingdom: Chapman & Hall; 1980. p. 63-104.

23. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. J Bacteriol. 2010;192(19):5002-17.

24. Asikainen S, Chen C. Oral ecology and person-to-person transmission of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. Periodontol 2000. 1999;20:65-81.

25. Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. Trends Immunol. 2014;35(1):3-11.

26. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. Nat Rev Microbiol. 2010;8(7):481-90.

27. Theilade E. The non-specific theory in microbial etiology of inflammatory periodontal diseases. J Clin Periodontol. 1986;13(10):905-11.

28. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998;25(2):134-44.

29. Curtis MA, Zenobia C, Darveau RP. The relationship of the oral microbiotia to periodontal health and disease. Cell Host Microbe. 2011;10(4):302-6.

30. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol. 2012;27(6):409-19.

31. Meyle J, Chapple I. Molecular aspects of the pathogenesis of periodontitis. Periodontol 2000. 2015;69(1):7-17.

32. Madianos PN, Bobetsis YA, Kinane DF. Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. J Clin Periodontol. 2005;32 Suppl 6:57-71.

33. Kornman KS, Page RC, Tonetti MS. The host response to the microbial challenge in periodontitis: assembling the players. Periodontol 2000. 1997;14:33-53.

34. Bage T, Kats A, Lopez BS, Morgan G, Nilsson G, Burt I, et al. Expression of prostaglandin E synthases in periodontitis immunolocalization and cellular regulation. Am J Pathol. 2011;178(4):1676-88.

35. Phipps RP, Borrello MA, Blieden TM. Fibroblast heterogeneity in the periodontium and other tissues. J Periodontal Res. 1997;32(1 Pt 2):159-65.

36. Hernandez M, Dutzan N, Garcia-Sesnich J, Abusleme L, Dezerega A, Silva N, et al. Host-pathogen interactions in progressive chronic periodontitis. J Dent Res. 2011;90(10):1164-70.

37. Birkedal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. J Periodontal Res. 1993;28(6 Pt 2):500-10.

38. Asai Y, Ohyama Y, Gen K, Ogawa T. Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. Infect Immun. 2001;69(12):7387-95.

39. Miyauchi M, Sato S, Kitagawa S, Hiraoka M, Kudo Y, Ogawa I, et al. Cytokine expression in rat molar gingival periodontal tissues after topical application of lipopolysaccharide. Histochem Cell Biol. 2001;116(1):57-62.

40. Huang GT, Haake SK, Kim JW, Park NH. Differential expression of interleukin-8 and intercellular adhesion molecule-1 by human gingival epithelial cells in response to Actinobacillus actinomycetemcomitans or Porphyromonas gingivalis infection. Oral Microbiol Immunol. 1998;13(5):301-9.

41. Groeger SE, Meyle J. Epithelial barrier and oral bacterial infection. Periodontol 2000. 2015;69(1):46-67.

42. Suchett-Kaye G, Morrier JJ, Barsotti O. Interactions between non-immune host cells and the immune system during periodontal disease: role of the gingival keratinocyte. Crit Rev Oral Biol Med. 1998;9(3):292-305.

43. Tonetti MS, Imboden MA, Gerber L, Lang NP, Laissue J, Mueller C. Localized expression of mRNA for phagocyte-specific chemotactic cytokines in human periodontal infections. Infect Immun. 1994;62(9):4005-14.

44. Tonetti MS, Imboden MA, Lang NP. Neutrophil migration into the gingival sulcus is associated with transepithelial gradients of interleukin-8 and ICAM-1. J Periodontol. 1998;69(10):1139-47.

45. Ohlrich EJ, Cullinan MP, Seymour GJ. The immunopathogenesis of periodontal disease. Aust Dent J. 2009;54 Suppl 1:S2-10.

46. Hovav AH. Dendritic cells of the oral mucosa. Mucosal Immunol. 2014;7(1):27-37.

47. Nussbaum G, Shapira L. How has neutrophil research improved our understanding of periodontal pathogenesis? J Clin Periodontol. 2011;38 Suppl 11:49-59.

48. Ryder MI. Comparison of neutrophil functions in aggressive and chronic periodontitis. Periodontol 2000. 2010;53:124-37.

49. Di Benedetto A, Gigante I, Colucci S, Grano M. Periodontal disease: linking the primary inflammation to bone loss. Clin Dev Immunol. 2013;2013:503754.

50. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol. 2008;8(5):349-61.

51. Van Dyke TE, Serhan CN. Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. J Dent Res. 2003;82(2):82-90.

52. Belting CM, Hiniker J, Dummett CO. Influence of diabetes mellitus on the severity of periodontal disease. J Periodontol. 1964;35(6):476-80.

53. Lalla E, Papapanou PN. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. Nat Rev Endocrinol. 2011;7(12):738-48.

54. Mealey BL, Oates TW, American Academy of P. Diabetes mellitus and periodontal diseases. J Periodontol. 2006;77(8):1289-303.

55. Taylor GW, Borgnakke WS. Periodontal disease: associations with diabetes, glycemic control and complications. Oral Dis. 2008;14(3):191-203.

56. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005;54(6):1615-25.

57. Preshaw PM, Alba AL, Herrera D, Jepsen S, Konstantinidis A, Makrilakis K, et al. Periodontitis and diabetes: a two-way relationship. Diabetologia. 2012;55(1):21-31.

58. Taylor GW. Bidirectional interrelationships between diabetes and periodontal diseases: an epidemiologic perspective. Ann Periodontol. 2001;6(1):99-112.

59. Bahekar AA, Singh S, Saha S, Molnar J, Arora R. The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: a meta-analysis. Am Heart J. 2007;154(5):830-7.

60. Friedewald VE, Kornman KS, Beck JD, Genco R, Goldfine A, Libby P, et al. The American Journal of Cardiology and Journal of Periodontology editors' consensus: periodontitis and atherosclerotic cardiovascular disease. J Periodontol. 2009;80(7):1021-32.

61. Kebschull M, Demmer RT, Papapanou PN. "Gum bug, leave my heart alone!"--epidemiologic and mechanistic evidence linking periodontal infections and atherosclerosis. J Dent Res. 2010;89(9):879-902.

62. Offenbacher S, Beck JD, Moss K, Mendoza L, Paquette DW, Barrow DA, et al. Results from the Periodontitis and Vascular Events (PAVE) Study: a pilot multicentered, randomized, controlled trial to study effects of periodontal therapy in a secondary prevention model of cardiovascular disease. J Periodontol. 2009;80(2):190-201.

63. Tonetti MS, D'Aiuto F, Nibali L, Donald A, Storry C, Parkar M, et al. Treatment of periodontitis and endothelial function. N Engl J Med. 2007;356(9):911-20.

64. Carrion J, Scisci E, Miles B, Sabino GJ, Zeituni AE, Gu Y, et al. Microbial carriage state of peripheral blood dendritic cells (DCs) in chronic periodontitis influences DC differentiation, atherogenic potential. J Immunol. 2012;189(6):3178-87.

65. Chiu B. Multiple infections in carotid atherosclerotic plaques. Am Heart J. 1999;138(5 Pt 2):S534-6.

66. Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. Identification of periodontal pathogens in atheromatous plaques. J Periodontol. 2000;71(10):1554-60.

67. Kozarov EV, Dorn BR, Shelburne CE, Dunn WA, Jr., Progulske-Fox A. Human atherosclerotic plaque contains viable invasive Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. Arterioscler Thromb Vasc Biol. 2005;25(3):e17-8.

68. Reyes L, Herrera D, Kozarov E, Rolda S, Progulske-Fox A. Periodontal bacterial invasion and infection: contribution to atherosclerotic pathology. J Periodontol. 2013;84(4 Suppl):S30-50.

69. Zelkha SA, Freilich RW, Amar S. Periodontal innate immune mechanisms relevant to atherosclerosis and obesity. Periodontol 2000. 2010;54(1):207-21.

70. Genco RJ, Van Dyke TE. Prevention: Reducing the risk of CVD in patients with periodontitis. Nat Rev Cardiol. 2010;7(9):479-80.

71. Tonetti MS. Periodontitis and risk for atherosclerosis: an update on intervention trials. J Clin Periodontol. 2009;36 Suppl 10:15-9.

72. Leech MT, Bartold PM. The association between rheumatoid arthritis and periodontitis. Best Pract Res Clin Rheumatol. 2015;29(2):189-201.

73. Catrina AI, Ytterberg AJ, Reynisdottir G, Malmstrom V, Klareskog L. Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. Nat Rev Rheumatol. 2014;10(11):645-53.

74. de Pablo P, Dietrich T, McAlindon TE. Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population. J Rheumatol. 2008;35(1):70-6.

75. Gully N, Bright R, Marino V, Marchant C, Cantley M, Haynes D, et al. Porphyromonas gingivalis peptidylarginine deiminase, a key contributor in the pathogenesis of experimental periodontal disease and experimental arthritis. PLoS One. 2014;9(6):e100838.

76. Gyorgy B, Toth E, Tarcsa E, Falus A, Buzas EI. Citrullination: a posttranslational modification in health and disease. Int J Biochem Cell Biol. 2006;38(10):1662-77.

77. Joseph R, Rajappan S, Nath SG, Paul BJ. Association between chronic periodontitis and rheumatoid arthritis: a hospital-based case-control study. Rheumatol Int. 2013;33(1):103-9.

78. Lundberg K, Kinloch A, Fisher BA, Wegner N, Wait R, Charles P, et al. Antibodies to citrullinated alpha-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase. Arthritis Rheum. 2008;58(10):3009-19.

79. Paju S, Scannapieco FA. Oral biofilms, periodontitis, and pulmonary infections. Oral Dis. 2007;13(6):508-12.

80. Pratesi F, Petit Teixeira E, Sidney J, Michou L, Puxeddu I, Sette A, et al. HLA shared epitope and ACPA: just a marker or an active player? Autoimmun Rev. 2013;12(12):1182-7.

81. Pyrc K, Milewska A, Kantyka T, Sroka A, Maresz K, Koziel J, et al. Inactivation of epidermal growth factor by Porphyromonas gingivalis as a potential mechanism for periodontal tissue damage. Infect Immun. 2013;81(1):55-64.

82. Scher JU, Bretz WA, Abramson SB. Periodontal disease and subgingival microbiota as contributors for rheumatoid arthritis pathogenesis: modifiable risk factors? Curr Opin Rheumatol. 2014;26(4):424-9.

83. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One. 2012;7(5):e35296.

84. Suwannalai P, Trouw LA, Toes RE, Huizinga TW. Anti-citrullinated protein antibodies (ACPA) in early rheumatoid arthritis. Mod Rheumatol. 2012;22(1):15-20.

85. Linden GJ, Herzberg MC, working group 4 of the joint EFPAAPw. Periodontitis and systemic diseases: a record of discussions of working group 4 of the Joint EFP/AAP

Workshop on Periodontitis and Systemic Diseases. J Periodontol. 2013;84(4 Suppl):S20-3.

86. Eriksson K, Nise L, Kats A, Luttropp E, Catrina AI, Askling J, et al. Prevalence of Periodontitis in Patients with Established Rheumatoid Arthritis: A Swedish Population Based Case-Control Study. PLoS One. 2016;11(5):e0155956.

87. Farquharson D, Butcher JP, Culshaw S. Periodontitis, Porphyromonas, and the pathogenesis of rheumatoid arthritis. Mucosal Immunol. 2012;5(2):112-20.

88. Martinez-Martinez RE, Abud-Mendoza C, Patino-Marin N, Rizo-Rodriguez JC, Little JW, Loyola-Rodriguez JP. Detection of periodontal bacterial DNA in serum and synovial fluid in refractory rheumatoid arthritis patients. J Clin Periodontol. 2009;36(12):1004-10.

89. Moen K, Brun JG, Valen M, Skartveit L, Eribe EK, Olsen I, et al. Synovial inflammation in active rheumatoid arthritis and psoriatic arthritis facilitates trapping of a variety of oral bacterial DNAs. Clin Exp Rheumatol. 2006;24(6):656-63.

90. van de Stadt LA, de Koning MH, van de Stadt RJ, Wolbink G, Dijkmans BA, Hamann D, et al. Development of the anti-citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. Arthritis Rheum. 2011;63(11):3226-33.

91. Brito F, de Barros FC, Zaltman C, Carvalho AT, Carneiro AJ, Fischer RG, et al. Prevalence of periodontitis and DMFT index in patients with Crohn's disease and ulcerative colitis. J Clin Periodontol. 2008;35(6):555-60.

92. Flemmig TF, Shanahan F, Miyasaki KT. Prevalence and severity of periodontal disease in patients with inflammatory bowel disease. J Clin Periodontol. 1991;18(9):690-7.

93. Grossner-Schreiber B, Fetter T, Hedderich J, Kocher T, Schreiber S, Jepsen S. Prevalence of dental caries and periodontal disease in patients with inflammatory bowel disease: a case-control study. J Clin Periodontol. 2006;33(7):478-84.

94. Habashneh RA, Khader YS, Alhumouz MK, Jadallah K, Ajlouni Y. The association between inflammatory bowel disease and periodontitis among Jordanians: a case-control study. J Periodontal Res. 2012;47(3):293-8.

95. Koutsochristou V, Zellos A, Dimakou K, Panayotou I, Siahanidou S, Roma-Giannikou E, et al. Dental Caries and Periodontal Disease in Children and Adolescents with Inflammatory Bowel Disease: A Case-Control Study. Inflamm Bowel Dis. 2015;21(8):1839-46.

96. Vavricka SR, Manser CN, Hediger S, Vogelin M, Scharl M, Biedermann L, et al. Periodontitis and gingivitis in inflammatory bowel disease: a case-control study. Inflamm Bowel Dis. 2013;19(13):2768-77.

97. Lira-Junior R, Figueredo CM. Periodontal and inflammatory bowel diseases: Is there evidence of complex pathogenic interactions? World J Gastroenterol. 2016;22(35):7963-72.

98. Brandtzaeg P. Inflammatory bowel disease: clinics and pathology. Do inflammatory bowel disease and periodontal disease have similar immunopathogeneses? Acta Odontol Scand. 2001;59(4):235-43.

99. Ruthruff B. Clinical review of Crohn's disease. J Am Acad Nurse Pract. 2007;19(8):392-7.

100. Ayangco L, Rogers RS, 3rd, Sheridan PJ. Pyostomatitis vegetans as an early sign of reactivation of Crohn's disease: a case report. J Periodontol. 2002;73(12):1512-6.

101. Lankarani KB, Sivandzadeh GR, Hassanpour S. Oral manifestation in inflammatory bowel disease: a review. World J Gastroenterol. 2013;19(46):8571-9.

102. Lourenco SV, Hussein TP, Bologna SB, Sipahi AM, Nico MM. Oral manifestations of inflammatory bowel disease: a review based on the observation of six cases. J Eur Acad Dermatol Venereol. 2010;24(2):204-7.

103. Pittock S, Drumm B, Fleming P, McDermott M, Imrie C, Flint S, et al. The oral cavity in Crohn's disease. J Pediatr. 2001;138(5):767-71.

104. Jaedicke KM, Preshaw PM, Taylor JJ. Salivary cytokines as biomarkers of periodontal diseases. Periodontol 2000. 2016;70(1):164-83.

105. Chaudhari AU, Byakod GN, Waghmare PF, Karhadkar VM. Correlation of levels of interleukin-1beta in gingival crevicular fluid to the clinical parameters of chronic periodontitis. J Contemp Dent Pract. 2011;12(1):52-9.

106. Engebretson SP, Grbic JT, Singer R, Lamster IB. GCF IL-1beta profiles in periodontal disease. J Clin Periodontol. 2002;29(1):48-53.

107. Gursoy UK, Kononen E, Pussinen PJ, Tervahartiala T, Hyvarinen K, Suominen AL, et al. Use of host- and bacteria-derived salivary markers in detection of periodontitis: a cumulative approach. Dis Markers. 2011;30(6):299-305.

108. Stashenko P, Dewhirst FE, Peros WJ, Kent RL, Ago JM. Synergistic interactions between interleukin 1, tumor necrosis factor, and lymphotoxin in bone resorption. J Immunol. 1987;138(5):1464-8.

109. Shimada Y, Komatsu Y, Ikezawa-Suzuki I, Tai H, Sugita N, Yoshie H. The effect of periodontal treatment on serum leptin, interleukin-6, and C-reactive protein. J Periodontol. 2010;81(8):1118-23.

110. Schierano G, Bellone G, Cassarino E, Pagano M, Preti G, Emanuelli G. Transforming growth factor-beta and interleukin 10 in oral implant sites in humans. J Dent Res. 2003;82(6):428-32.

111. Liskmann S, Vihalemm T, Salum O, Zilmer K, Fischer K, Zilmer M. Correlations between clinical parameters and interleukin-6 and interleukin-10 levels in saliva from totally edentulous patients with peri-implant disease. Int J Oral Maxillofac Implants. 2006;21(4):543-50.

112. Kwan Tat S, Padrines M, Theoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev. 2004;15(1):49-60.

113. Geivelis M, Turner DW, Pederson ED, Lamberts BL. Measurements of interleukin-6 in gingival crevicular fluid from adults with destructive periodontal disease. J Periodontol. 1993;64(10):980-3.

114. Costa PP, Trevisan GL, Macedo GO, Palioto DB, Souza SL, Grisi MF, et al. Salivary interleukin-6, matrix metalloproteinase-8, and osteoprotegerin in patients with periodontitis and diabetes. J Periodontol. 2010;81(3):384-91.

115. Fine DH, Markowitz K, Furgang D, Fairlie K, Ferrandiz J, Nasri C, et al. Macrophage inflammatory protein-1alpha: a salivary biomarker of bone loss in a longitudinal

cohort study of children at risk for aggressive periodontal disease? J Periodontol. 2009;80(1):106-13.

116. Al-Sabbagh M, Alladah A, Lin Y, Kryscio RJ, Thomas MV, Ebersole JL, et al. Bone remodeling-associated salivary biomarker MIP-1alpha distinguishes periodontal disease from health. J Periodontal Res. 2012;47(3):389-95.

117. Sanchez GA, Miozza VA, Delgado A, Busch L. Salivary IL-1beta and PGE2 as biomarkers of periodontal status, before and after periodontal treatment. J Clin Periodontol. 2013;40(12):1112-7.

118. Sexton WM, Lin Y, Kryscio RJ, Dawson DR, 3rd, Ebersole JL, Miller CS. Salivary biomarkers of periodontal disease in response to treatment. J Clin Periodontol. 2011;38(5):434-41.

119. Miller CS, King CP, Jr., Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing periodontal disease: a cross-sectional study. J Am Dent Assoc. 2006;137(3):322-9.

120. Gursoy UK, Kononen E, Pradhan-Palikhe P, Tervahartiala T, Pussinen PJ, Suominen-Taipale L, et al. Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. J Clin Periodontol. 2010;37(6):487-93.

121. Kinney JS, Morelli T, Braun T, Ramseier CA, Herr AE, Sugai JV, et al. Saliva/pathogen biomarker signatures and periodontal disease progression. J Dent Res. 2011;90(6):752-8.

122. Miller CS, Foley JD, Bailey AL, Campell CL, Humphries RL, Christodoulides N, et al. Current developments in salivary diagnostics. Biomark Med. 2010;4(1):171-89.

123. Ramseier CA, Kinney JS, Herr AE, Braun T, Sugai JV, Shelburne CA, et al. Identification of pathogen and host-response markers correlated with periodontal disease. J Periodontol. 2009;80(3):436-46.

124. Sorsa T, Tjaderhane L, Konttinen YT, Lauhio A, Salo T, Lee HM, et al. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. Ann Med. 2006;38(5):306-21.

125. Chambers DA, Crawford JM, Mukherjee S, Cohen RL. Aspartate aminotransferase increases in crevicular fluid during experimental periodontitis in beagle dogs. J Periodontol. 1984;55(9):526-30.

126. Nomura Y, Shimada Y, Hanada N, Numabe Y, Kamoi K, Sato T, et al. Salivary biomarkers for predicting the progression of chronic periodontitis. Arch Oral Biol. 2012;57(4):413-20.

127. Nomura Y, Tamaki Y, Tanaka T, Arakawa H, Tsurumoto A, Kirimura K, et al. Screening of periodontitis with salivary enzyme tests. J Oral Sci. 2006;48(4):177-83.

128. Page RC. Host response tests for diagnosing periodontal diseases. J Periodontol. 1992;63(4 Suppl):356-66.

129. Todorovic T, Dozic I, Vicente-Barrero M, Ljuskovic B, Pejovic J, Marjanovic M, et al. Salivary enzymes and periodontal disease. Med Oral Patol Oral Cir Bucal. 2006;11(2):E115-9.

130. Totan A, Greabu M, Totan C, Spinu T. Salivary aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase: possible markers in periodontal diseases? Clin Chem Lab Med. 2006;44(5):612-5.

131. Jin Q, Cirelli JA, Park CH, Sugai JV, Taba M, Jr., Kostenuik PJ, et al. RANKL inhibition through osteoprotegerin blocks bone loss in experimental periodontitis. J Periodontol. 2007;78(7):1300-8.

132. Ng PY, Donley M, Hausmann E, Hutson AD, Rossomando EF, Scannapieco FA. Candidate salivary biomarkers associated with alveolar bone loss: cross-sectional and in vitro studies. FEMS Immunol Med Microbiol. 2007;49(2):252-60.

133. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol. 1992;63(4 Suppl):322-31.

134. Korte DL, Kinney J. Personalized medicine: an update of salivary biomarkers for periodontal diseases. Periodontol 2000. 2016;70(1):26-37.

135. de Lima CL, Acevedo AC, Grisi DC, Taba M, Jr., Guerra E, De Luca Canto G. Host-derived salivary biomarkers in diagnosing periodontal disease: systematic review and meta-analysis. J Clin Periodontol. 2016;43(6):492-502.

136. Ebersole JL, Schuster JL, Stevens J, Dawson D, 3rd, Kryscio RJ, Lin Y, et al. Patterns of salivary analytes provide diagnostic capacity for distinguishing chronic adult periodontitis from health. J Clin Immunol. 2013;33(1):271-9.

137. Gursoy UK, Kononen E, Uitto VJ, Pussinen PJ, Hyvarinen K, Suominen-Taipale L, et al. Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis. J Clin Periodontol. 2009;36(11):922-7.

138. Salminen A, Gursoy UK, Paju S, Hyvarinen K, Mantyla P, Buhlin K, et al. Salivary biomarkers of bacterial burden, inflammatory response, and tissue destruction in periodontitis. J Clin Periodontol. 2014;41(5):442-50.

139. Mantyla P, Stenman M, Kinane DF, Tikanoja S, Luoto H, Salo T, et al. Gingival crevicular fluid collagenase-2 (MMP-8) test stick for chair-side monitoring of periodontitis. J Periodontal Res. 2003;38(4):436-9.

140. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet. 2016;17(6):333-51.

141. Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X. Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. PLoS One. 2014;9(1):e78644.

142. McPherson JD. A defining decade in DNA sequencing. Nat Methods. 2014;11(10):1003-5.

143. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, et al. Accurate whole human genome sequencing using reversible terminator chemistry. Nature. 2008;456(7218):53-9.

144. Fu X, Fu N, Guo S, Yan Z, Xu Y, Hu H, et al. Estimating accuracy of RNA-Seq and microarrays with proteomics. BMC Genomics. 2009;10:161.

145. Sirbu A, Kerr G, Crane M, Ruskin HJ. RNA-Seq vs dual- and single-channel microarray data: sensitivity analysis for differential expression and clustering. PLoS One. 2012;7(12):e50986.

146. Wang C, Gong B, Bushel PR, Thierry-Mieg J, Thierry-Mieg D, Xu J, et al. The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. Nat Biotechnol. 2014;32(9):926-32.

147. Xu X, Zhang Y, Williams J, Antoniou E, McCombie WR, Wu S, et al. Parallel comparison of Illumina RNA-Seq and Affymetrix microarray platforms on transcriptomic profiles generated from 5-aza-deoxy-cytidine treated HT-29 colon cancer cells and simulated datasets. BMC Bioinformatics. 2013;14 Suppl 9:S1.

148. Hashimshony T, Wagner F, Sher N, Yanai I. CEL-Seq: single-cell RNA-Seq by multiplexed linear amplification. Cell Rep. 2012;2(3):666-73.

149. Islam S, Kjallquist U, Moliner A, Zajac P, Fan JB, Lonnerberg P, et al. Highly multiplexed and strand-specific single-cell RNA 5' end sequencing. Nat Protoc. 2012;7(5):813-28.

150. Ramskold D, Luo S, Wang YC, Li R, Deng Q, Faridani OR, et al. Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. Nat Biotechnol. 2012;30(8):777-82.

151. Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, et al. mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods. 2009;6(5):377-82.

152. Petropoulos S, Edsgard D, Reinius B, Deng Q, Panula SP, Codeluppi S, et al. Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos. Cell. 2016;165(4):1012-26.

153. Blakeley P, Fogarty NM, del Valle I, Wamaitha SE, Hu TX, Elder K, et al. Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. Development. 2015;142(18):3151-65.

154. Tirosh I, Izar B, Prakadan SM, Wadsworth MH, 2nd, Treacy D, Trombetta JJ, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science. 2016;352(6282):189-96.

155. Zheng C, Zheng L, Yoo JK, Guo H, Zhang Y, Guo X, et al. Landscape of Infiltrating T Cells in Liver Cancer Revealed by Single-Cell Sequencing. Cell. 2017;169(7):1342-56 e16.

156. Zeisel A, Munoz-Manchado AB, Codeluppi S, Lonnerberg P, La Manno G, Jureus A, et al. Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. Science. 2015;347(6226):1138-42.

157. Usoskin D, Furlan A, Islam S, Abdo H, Lonnerberg P, Lou D, et al. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci. 2015;18(1):145-53.

158. Stubbington MJT, Lonnberg T, Proserpio V, Clare S, Speak AO, Dougan G, et al. T cell fate and clonality inference from single-cell transcriptomes. Nat Methods. 2016;13(4):329-32.

159. Shalek AK, Satija R, Shuga J, Trombetta JJ, Gennert D, Lu D, et al. Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature. 2014;510(7505):363-9.

160. Chen J, Suo S, Tam PP, Han JJ, Peng G, Jing N. Spatial transcriptomic analysis of cryosectioned tissue samples with Geo-seq. Nat Protoc. 2017;12(3):566-80.

161. Farris S, Wang Y, Ward JM, Dudek SM. Optimized Method for Robust Transcriptome Profiling of Minute Tissues Using Laser Capture Microdissection and Low-Input RNA-Seq. Front Mol Neurosci. 2017;10:185.

162. Zechel S, Zajac P, Lonnerberg P, Ibanez CF, Linnarsson S. Topographical transcriptome mapping of the mouse medial ganglionic eminence by spatially resolved RNA-seq. Genome Biol. 2014;15(10):486.

163. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. Science. 2015;348(6233):aaa6090.

164. Lee JH. Quantitative approaches for investigating the spatial context of gene expression. Wiley Interdiscip Rev Syst Biol Med. 2017;9(2).

165. Levsky JM, Shenoy SM, Pezo RC, Singer RH. Single-cell gene expression profiling. Science. 2002;297(5582):836-40.

166. Lubeck E, Coskun AF, Zhiyentayev T, Ahmad M, Cai L. Single-cell in situ RNA profiling by sequential hybridization. Nat Methods. 2014;11(4):360-1.

167. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. Nat Methods. 2008;5(10):877-9.

168. Lee JH, Daugharthy ER, Scheiman J, Kalhor R, Ferrante TC, Terry R, et al. Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues. Nat Protoc. 2015;10(3):442-58.

169. Ke R, Mignardi M, Pacureanu A, Svedlund J, Botling J, Wahlby C, et al. In situ sequencing for RNA analysis in preserved tissue and cells. Nat Methods. 2013;10(9):857-60.

170. Demmer RT, Behle JH, Wolf DL, Handfield M, Kebschull M, Celenti R, et al. Transcriptomes in healthy and diseased gingival tissues. J Periodontol. 2008;79(11):2112-24.

171. Kebschull M, Guarnieri P, Demmer RT, Boulesteix AL, Pavlidis P, Papapanou PN. Molecular differences between chronic and aggressive periodontitis. J Dent Res. 2013;92(12):1081-8.

172. Kebschull M, Demmer RT, Grun B, Guarnieri P, Pavlidis P, Papapanou PN. Gingival tissue transcriptomes identify distinct periodontitis phenotypes. J Dent Res. 2014;93(5):459-68.

173. Davanian H, Stranneheim H, Bage T, Lagervall M, Jansson L, Lundeberg J, et al. Gene expression profiles in paired gingival biopsies from periodontitis-affected and healthy tissues revealed by massively parallel sequencing. PLoS One. 2012;7(9):e46440.

174. Becker ST, Beck-Broichsitter BE, Graetz C, Dorfer CE, Wiltfang J, Hasler R. Peri-implantitis versus periodontitis: functional differences indicated by transcriptome profiling. Clin Implant Dent Relat Res. 2014;16(3):401-11.

175. Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD, et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. ISME J. 2013;7(5):1016-25.

176. Griffen AL, Beall CJ, Campbell JH, Firestone ND, Kumar PS, Yang ZK, et al. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. ISME J. 2012;6(6):1176-85.

177. Mira A, Simon-Soro A, Curtis MA. Role of microbial communities in the pathogenesis of periodontal diseases and caries. J Clin Periodontol. 2017;44 Suppl 18:S23-S38.

178. Ge X, Rodriguez R, Trinh M, Gunsolley J, Xu P. Oral microbiome of deep and shallow dental pockets in chronic periodontitis. PLoS One. 2013;8(6):e65520.

179. Li Y, He J, He Z, Zhou Y, Yuan M, Xu X, et al. Phylogenetic and functional gene structure shifts of the oral microbiomes in periodontitis patients. ISME J. 2014;8(9):1879-91.

180. Kirst ME, Li EC, Alfant B, Chi YY, Walker C, Magnusson I, et al. Dysbiosis and alterations in predicted functions of the subgingival microbiome in chronic periodontitis. Appl Environ Microbiol. 2015;81(2):783-93.

181. Belstrom D, Fiehn NE, Nielsen CH, Kirkby N, Twetman S, Klepac-Ceraj V, et al. Differences in bacterial saliva profile between periodontitis patients and a control cohort. J Clin Periodontol. 2014;41(2):104-12.

182. Takeshita T, Kageyama S, Furuta M, Tsuboi H, Takeuchi K, Shibata Y, et al. Bacterial diversity in saliva and oral health-related conditions: the Hisayama Study. Sci Rep. 2016;6:22164.

183. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A. 1991;88(16):7276-80.

184. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol. 2006;7:3.

185. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.

186. Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). J Chemometrics. 2002;16(3):119-28.

187. Li J, Tibshirani R. Finding consistent patterns: a nonparametric approach for identifying differential expression in RNA-Seq data. Stat Methods Med Res. 2013;22(5):519-36.

188. Cangemi C, Skov V, Poulsen MK, Funder J, Twal WO, Gall MA, et al. Fibulin-1 is a marker for arterial extracellular matrix alterations in type 2 diabetes. Clin Chem. 2011;57(11):1556-65.

189. Folkersen L, Persson J, Ekstrand J, Agardh HE, Hansson GK, Gabrielsen A, et al. Prediction of ischemic events on the basis of transcriptomic and genomic profiling in patients undergoing carotid endarterectomy. Mol Med. 2012;18:669-75.

190. Noble CL, Abbas AR, Cornelius J, Lees CW, Ho GT, Toy K, et al. Regional variation in gene expression in the healthy colon is dysregulated in ulcerative colitis. Gut. 2008;57(10):1398-405.

191. Sikorski K, Wesoly J, Bluyssen HA. Data mining of atherosclerotic plaque transcriptomes predicts STAT1-dependent inflammatory signal integration in vascular disease. Int J Mol Sci. 2014;15(8):14313-31.

192. Ungethuem U, Haeupl T, Witt H, Koczan D, Krenn V, Huber H, et al. Molecular signatures and new candidates to target the pathogenesis of rheumatoid arthritis. Physiol Genomics. 2010;42A(4):267-82.

193. Le Cao KA, Boitard S, Besse P. Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems. BMC Bioinformatics. 2011;12:253.

194. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.

195. Liaw A, Wiener M. Classification and Regression by randomForest. R News. 2002;2(3):18-22.

196. Van Der Maaten L. Accelerating t-SNE using tree-based algorithms. Journal of machine learning research. 2014;15(1):3221-45.

197. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

198. Vitale Shreve KR, Udell MA. What's inside your cat's head? A review of cat (Felis silvestris catus) cognition research past, present and future. Anim Cogn. 2015;18(6):1195-206.

199. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. Mucosal Immunol. 2008;1(3):183-97.

200. Fischer BM, Cuellar JG, Diehl ML, deFreytas AM, Zhang J, Carraway KL, et al. Neutrophil elastase increases MUC4 expression in normal human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2003;284(4):L671-9.

201. Iwashita J, Sato Y, Sugaya H, Takahashi N, Sasaki H, Abe T. mRNA of MUC2 is stimulated by IL-4, IL-13 or TNF-alpha through a mitogen-activated protein kinase pathway in human colon cancer cells. Immunol Cell Biol. 2003;81(4):275-82.

202. Kim YD, Kwon EJ, Kwon TK, Baek SH, Song SY, Suh JS. Regulation of IL-1beta-mediated MUC2 gene in NCI-H292 human airway epithelial cells. Biochem Biophys Res Commun. 2000;274(1):112-6.

203. Smirnova MG, Birchall JP, Pearson JP. TNF-alpha in the regulation of MUC5AC secretion: some aspects of cytokine-induced mucin hypersecretion on the in vitro model. Cytokine. 2000;12(11):1732-6.

204. Smirnova MG, Guo L, Birchall JP, Pearson JP. LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells. Cell Immunol. 2003;221(1):42-9.

205. Smirnova MG, Kiselev SL, Birchall JP, Pearson JP. Up-regulation of mucin secretion in HT29-MTX cells by the pro-inflammatory cytokines tumor necrosis factor-alpha and interleukin-6. Eur Cytokine Netw. 2001;12(1):119-25.

206. Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, et al. Interleukin-1 beta and tumor necrosis factor-alpha induce MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. J Biol Chem. 2003;278(26):23243-50.

207. Kufe DW. Mucins in cancer: function, prognosis and therapy. Nat Rev Cancer. 2009;9(12):874-85.

208. Mukhopadhyay P, Lakshmanan I, Ponnusamy MP, Chakraborty S, Jain M, Pai P, et al. MUC4 overexpression augments cell migration and metastasis through EGFR family proteins in triple negative breast cancer cells. PLoS One. 2013;8(2):e54455.

209. Sobhia ME, Grewal BK, Ml SP, Patel J, Kaur A, Haokip T, et al. Protein kinase C inhibitors: a patent review (2008 - 2009). Expert Opin Ther Pat. 2013;23(10):1297-315.

210. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim Biophys Acta. 2010;1803(1):55-71.

211. Silva N, Abusleme L, Bravo D, Dutzan N, Garcia-Sesnich J, Vernal R, et al. Host response mechanisms in periodontal diseases. J Appl Oral Sci. 2015;23(3):329-55.

212. Makela M, Larjava H, Pirila E, Maisi P, Salo T, Sorsa T, et al. Matrix metalloproteinase 2 (gelatinase A) is related to migration of keratinocytes. Exp Cell Res. 1999;251(1):67-78.

213. Uitto VJ, Overall CM, McCulloch C. Proteolytic host cell enzymes in gingival crevice fluid. Periodontol 2000. 2003;31:77-104.

214. Hilkens J, Buijs F. Biosynthesis of MAM-6, an epithelial sialomucin. Evidence for involvement of a rare proteolytic cleavage step in the endoplasmic reticulum. J Biol Chem. 1988;263(9):4215-22.

215. Sheng ZQ, Hull SR, Carraway KL. Biosynthesis of the cell surface sialomucin complex of ascites 13762 rat mammary adenocarcinoma cells from a high molecular weight precursor. J Biol Chem. 1990;265(15):8505-10.

216. Williams SJ, Wreschner DH, Tran M, Eyre HJ, Sutherland GR, McGuckin MA. Muc13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. J Biol Chem. 2001;276(21):18327-36.

217. Andrianifahanana M, Agrawal A, Singh AP, Moniaux N, van Seuningen I, Aubert JP, et al. Synergistic induction of the MUC4 mucin gene by interferon-gamma and retinoic acid in human pancreatic tumour cells involves a reprogramming of signalling pathways. Oncogene. 2005;24(40):6143-54.

218. Acquier AB, Pita AK, Busch L, Sanchez GA. Comparison of salivary levels of mucin and amylase and their relation with clinical parameters obtained from patients with aggressive and chronic periodontal disease. J Appl Oral Sci. 2015;23(3):288-94.

219. Sanchez GA, Miozza V, Delgado A, Busch L. Determination of salivary levels of mucin and amylase in chronic periodontitis patients. J Periodontal Res. 2011;46(2):221-7.

220. Sanchez GA, Miozza VA, Delgado A, Busch L. Relationship between salivary mucin or amylase and the periodontal status. Oral Dis. 2013;19(6):585-91.

221. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol. 2004;4(8):617-29.

222. Rathnayake N, Akerman S, Klinge B, Lundegren N, Jansson H, Tryselius Y, et al. Salivary biomarkers of oral health: a cross-sectional study. J Clin Periodontol. 2013;40(2):140-7.

223. Balbin M, Fueyo A, Knauper V, Pendas AM, Lopez JM, Jimenez MG, et al. Collagenase 2 (MMP-8) expression in murine tissue-remodeling processes. Analysis

of its potential role in postpartum involution of the uterus. J Biol Chem. 1998;273(37):23959-68.

224. Ding Y, Kantarci A, Badwey JA, Hasturk H, Malabanan A, Van Dyke TE. Phosphorylation of pleckstrin increases proinflammatory cytokine secretion by mononuclear phagocytes in diabetes mellitus. J Immunol. 2007;179(1):647-54.

225. Hasturk H, Kantarci A, Van Dyke TE. Oral inflammatory diseases and systemic inflammation: role of the macrophage. Front Immunol. 2012;3:118.

226. Brumell JH, Howard JC, Craig K, Grinstein S, Schreiber AD, Tyers M. Expression of the protein kinase C substrate pleckstrin in macrophages: association with phagosomal membranes. J Immunol. 1999;163(6):3388-95.

227. Liu B, Faller LL, Klitgord N, Mazumdar V, Ghodsi M, Sommer DD, et al. Deep sequencing of the oral microbiome reveals signatures of periodontal disease. PLoS One. 2012;7(6):e37919.

228. Abiko Y, Sato T, Mayanagi G, Takahashi N. Profiling of subgingival plaque biofilm microflora from periodontally healthy subjects and from subjects with periodontitis using quantitative real-time PCR. J Periodontal Res. 2010;45(3):389-95.

229. Chen H, Liu Y, Zhang M, Wang G, Qi Z, Bridgewater L, et al. A Filifactor alocis-centered co-occurrence group associates with periodontitis across different oral habitats. Sci Rep. 2015;5:9053.

230. Colombo AP, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, et al. Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. J Periodontol. 2009;80(9):1421-32.

231. Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species associated with chronic periodontitis. J Dent Res. 2003;82(5):338-44.

232. Mayanagi G, Sato T, Shimauchi H, Takahashi N. Detection frequency of periodontitis-associated bacteria by polymerase chain reaction in subgingival and supragingival plaque of periodontitis and healthy subjects. Oral Microbiol Immunol. 2004;19(6):379-85.

233. Oliveira RR, Fermiano D, Feres M, Figueiredo LC, Teles FR, Soares GM, et al. Levels of Candidate Periodontal Pathogens in Subgingival Biofilm. J Dent Res. 2016;95(6):711-8.

234. Fine DH, Markowitz K, Fairlie K, Tischio-Bereski D, Ferrendiz J, Furgang D, et al. A consortium of Aggregatibacter actinomycetemcomitans, Streptococcus parasanguinis, and Filifactor alocis is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. J Clin Microbiol. 2013;51(9):2850-61.

235. Deng ZL, Szafranski SP, Jarek M, Bhuju S, Wagner-Dobler I. Dysbiosis in chronic periodontitis: Key microbial players and interactions with the human host. Sci Rep. 2017;7(1):3703.

236. Salminen A, Kopra KA, Hyvarinen K, Paju S, Mantyla P, Buhlin K, et al. Quantitative PCR analysis of salivary pathogen burden in periodontitis. Front Cell Infect Microbiol. 2015;5:69.

237. Marchesan JT, Morelli T, Moss K, Barros SP, Ward M, Jenkins W, et al. Association of Synergistetes and Cyclodipeptides with Periodontitis. J Dent Res. 2015;94(10):1425-31.

238. Camelo-Castillo AJ, Mira A, Pico A, Nibali L, Henderson B, Donos N, et al. Subgingival microbiota in health compared to periodontitis and the influence of smoking. Front Microbiol. 2015;6:119.

239. White UA, Stephens JM. The gp130 receptor cytokine family: regulators of adipocyte development and function. Curr Pharm Des. 2011;17(4):340-6.

240. Sims NA, Walsh NC. GP130 cytokines and bone remodelling in health and disease. BMB Rep. 2010;43(8):513-23.

241. Barkhordar RA, Hayashi C, Hussain MZ. Detection of interleukin-6 in human dental pulp and periapical lesions. Endod Dent Traumatol. 1999;15(1):26-7.

242. Johnson RB, Wood N, Serio FG. Interleukin-11 and IL-17 and the pathogenesis of periodontal disease. J Periodontol. 2004;75(1):37-43.

243. Lin SJ, Chen YL, Kuo MY, Li CL, Lu HK. Measurement of gp130 cytokines oncostatin M and IL-6 in gingival crevicular fluid of patients with chronic periodontitis. Cytokine. 2005;30(4):160-7.

244. Ogura N, Shibata Y, Kamino Y, Matsuda U, Hayakawa M, Oikawa T, et al. Stimulation of interleukin-6 production of periodontal ligament cells by Porphyromonas endodontalis lipopolysaccharide. Biochem Med Metab Biol. 1994;53(2):130-6.

245. Pradeep AR, S TM, Garima G, Raju A. Serum levels of oncostatin M (a gp 130 cytokine): an inflammatory biomarker in periodontal disease. Biomarkers. 2010;15(3):277-82.

246. Tsai CH, Huang FM, Chang YC. Immunohistochemical localization of oncostatin M in epithelialized apical periodontitis lesions. Int Endod J. 2008;41(9):772-6.

247. Quartermain L, Tailor H, Njenga S, Bhattacharjee P, Rao GG. Neonatal Granulicatella elegans bacteremia, London, UK. Emerg Infect Dis. 2013;19(7):1165-6.

248. Rathnayake N, Akerman S, Klinge B, Lundegren N, Jansson H, Tryselius Y, et al. Salivary biomarkers for detection of systemic diseases. PLoS One. 2013;8(4):e61356.

249. Guzeldemir-Akcakanat E, Sunnetci-Akkoyunlu D, Orucguney B, Cine N, Kan B, Yilmaz EB, et al. Gene-Expression Profiles in Generalized Aggressive Periodontitis: A Gene Network-Based Microarray Analysis. J Periodontol. 2016;87(1):58-65.

250. Kasar S, Kim J, Improgo R, Tiao G, Polak P, Haradhvala N, et al. Whole-genome sequencing reveals activation-induced cytidine deaminase signatures during indolent chronic lymphocytic leukaemia evolution. Nat Commun. 2015;6:8866.

251. Kanda M, Tanaka C, Kobayashi D, Tanaka H, Shimizu D, Shibata M, et al. Epigenetic suppression of the immunoregulator MZB1 is associated with the malignant phenotype of gastric cancer. Int J Cancer. 2016;139(10):2290-8.

252. Rosenbaum M, Andreani V, Kapoor T, Herp S, Flach H, Duchniewicz M, et al. MZB1 is a GRP94 cochaperone that enables proper immunoglobulin heavy chain biosynthesis upon ER stress. Genes Dev. 2014;28(11):1165-78.

253. Flach H, Rosenbaum M, Duchniewicz M, Kim S, Zhang SL, Cahalan MD, et al. Mzb1 protein regulates calcium homeostasis, antibody secretion, and integrin activation in innate-like B cells. Immunity. 2010;33(5):723-35.

254. Menetret JF, Hegde RS, Heinrich SU, Chandramouli P, Ludtke SJ, Rapoport TA, et al. Architecture of the ribosome-channel complex derived from native membranes. J Mol Biol. 2005;348(2):445-57.

255. Losfeld ME, Ng BG, Kircher M, Buckingham KJ, Turner EH, Eroshkin A, et al. A new congenital disorder of glycosylation caused by a mutation in SSR4, the signal sequence receptor 4 protein of the TRAP complex. Hum Mol Genet. 2014;23(6):1602-5.

256. Snapp EL, Reinhart GA, Bogert BA, Lippincott-Schwartz J, Hegde RS. The organization of engaged and quiescent translocons in the endoplasmic reticulum of mammalian cells. The Journal of cell biology. 2004;164(7):997-1007.

257. Singh H, Farouk M, Bose BB, Singh P. Novel genes underlying beta cell survival in metabolic stress. Bioinformation. 2013;9(1):37-41.

258. Navid F, Colbert RA. Causes and consequences of endoplasmic reticulum stress in rheumatic disease. Nat Rev Rheumatol. 2017;13(1):25-40.

259. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11(10):R106.

260. Edfors F, Danielsson F, Hallstrom BM, Kall L, Lundberg E, Ponten F, et al. Gene-specific correlation of RNA and protein levels in human cells and tissues. Mol Syst Biol. 2016;12(10):883.