From Department of Medicine Solna
Karolinska Institutet, Stockholm, Sweden

STUDIES OF PATHOPHYSIOLOGICAL MECHANISMS AND ORAL CAVITY MANIFESTATIONS IN CHRONIC INFLAMMATORY DISEASES

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Karolinska Institutet

Stockholm 2023

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Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2023

ISBN 978-91-8016-996-7
Cover illustration: *La Catrina* version 5.5.23 presents: "*Por la boca muere el pez"*. An illustration representing the oral cavity as a pathway to disease in the whole body. **By Guillermo Ruacho**

Studies of Pathophysiological Mechanisms and Oral Cavity Manifestations in Chronic Inflammatory Diseases

Thesis for Doctoral Degree (Ph.D.)

By

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Doctor of Dental Medicine

The thesis will be defended in public at Skandiasalen, Astrid Lindgrens Barnsjukhus, Karolinska University Hospital, Solna Stockholm, **5th of May 2023** at **9.00 am**

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A mis sobrinos:

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René, Gael,

Valentina,

Dahna, Ximena,

y Eloisa.

Me he perdido de muchas Aventuras con ustedes por vivir ésta.

"May God grant me to speak with judgment and to have thoughts worthy of what I have received, for he is the guide even of wisdom and the corrector of the wise. For both we and our words are in his hand, as are all understanding and skill in crafts. For it is he who gave me unerring knowledge of what exists…."

Wisdom 7: 15–17

Populärvetenskapligt sammanfattning

Systemisk Lupus Erythematosus (SLE) är en kronisk autoimmun sjukdom där 9 av 10 patienter är kvinnor. SLE överlappar delvis andra inflammatoriska sjukdomar; tex. ledgångsreumatism, *reumatoid artrit* (RA) avseende symptom och autoantikroppar. Dessa sjukdomar kräver upprepande besök och långvariga behandlingar med syfte att påverka patientens livskvalitet och sjukdomsprognos då det ännu inte finns någon botande behandling. I denna avhandling undersökte vi om man kan detektera inflammationsmarkörer associerade till SLE i munnen, och om dessa kan bidra till ökad förståelse för de immunologiska processer som orsakar autoimmunitet.

I **studie I** konstaterade vi att SS förekommer hos 1 av 5 SLE patienter, en högre frekvens än vad som presenterats i tidigare studier, 6–14%. Dessa fynd understryker vikten av munhålan och saliven vid SLE. Subgrupperna, SLE med och SLE utan sjögrens syndrome (SS) jämfördes kliniskt och utifrån laboratorieundersökningar och resultaten visar att potentiella biomarkörer för inflammation var högre i gruppen SLE med SS.

I **studie II** jämfördes biomarkörer för sjukdomsaktivitet i serum, saliv och urin från SLE patienter. Biomarkörnivåerna i saliv kunde särskilja SLE patienter från kontroller och de var associerade med högre sjukdomsaktivitet. Dessa resultat innebär en attraktiv möjlighet att följa sjukdomsaktiviteten vid systemiska sjukdomar på ett smidigare sätt.

I **studie III** undersöktes huruvida tandlossningsbakterier skapar infektioner som bidrar med utlösande faktorer till autoimmuna sjukdomar. Resultaten visar att immunsvarets svar mot en bakterie som förekommer vid parodontit, *arginine gingipain* (Rgp) IgG antikroppar, var relaterade till förekomsten av svår tandlossning men också till autoantikroppar som är vanliga vid RA och SLE.

I **studie IV** presenteras här en pilotstudie där vi undersökt salivprover för förekomst av immunglobuliner och antikroppar, som rutinmässigt mäts i blod. Enligt preliminära resultat återspeglar salivnivåerna mätningarna i blodet från SLE patienter med och utan SS. Det långsiktiga målet är att studera om och hur autoantikroppar i saliv kan användas för att diagnostisera eller förutse SS eller andra immunologiska aspekter.

Denna avhandling visar att munnen, och lokala sjukdomstillstånd där som PD samt saliven är viktiga för patienter med autoimmuna sjukdomar som SS, SLE *och* RA. Vi visar att det kan finnas möjligheter att mäta biomarkörer för sjukdomsaktivitet i saliv istället för blod. Vidare bidrar våra studier till ökad förstående för munnens roll för patogenesen av autoimmunitet. Dessa fynd öppnar en dörr av möjligheter till utveckling av sjukvården där man lättare och mindre invasivt kan diagnostisera och följa upp autoimmuna systemiska sjukdomar.

Divulgación científica

Lupus Eritematoso Sistémico (LES) es una enfermedad autoinmune con una prevalencia femenina de 1:9 y con características comunes a las de otras enfermedades inflamatorias como la artritis reumatoide (AR). El monitoreo de la actividad de la enfermedad (AE) ofrece a estos pacientes una mejora en su pronóstico y en su calidad de vida al no existir un tratamiento para erradicar esta enfermedad. En esta tesis analizamos el rol de la cavidad oral y su potencial para ofrecer indicadores de inflamación, así como los posibles mecanismos fisiopatológicos que puedan explicar la autoinmunidad.

En el **estudio I** demostramos que el síndrome de Sjögren´s (SS) afecta 1/4 de los pacientes con LES, lo cuál remarca el rol de la cavidad oral y las glándulas salivares en el LES. Los subgrupos obtenidos, con y sin presencia de SS, fueron comparados, y se identificaron posibles indicadores biológicos que fueron más elevados en el grupo de pacientes con SS.

En el **estudio II** comparamos indicadores de AE en muestras de orina, saliva y suero de pacientes con LES. El *factor estimulador de colonias -1,* el *factor de necrosis tumoral alfa, la proteína inducida por interferon-γ-10* y la *proteína quimio atractiva de monocitos-1,* así como *calprotectina* en saliva, son capaces de distinguir entre pacientes y controles, además de asociarse con la AE. Esto demuestra el potencial de la saliva como una alternativa al suero o a la orina.

En el **estudio III** investigamos la asociación de autoanticuerpos y anticuerpos contra la *arginina gingipaina (Rgp) IgG* con la enfermedad periodontal (EP) y su grado de severidad, en tres diferentes poblaciones. Los *anticuerpos antipéptidos cíclicos citrulinados (ACPA)* se asocian con la presencia de EP; y *ACPA* y los anticuerpos *dsDNA* asocian con anticuerpos contra *Rgp IgG*, los cuáles se asocian a su vez con el grado severo de la EP. Estos hallazgos apoyan el estudio de una vía oral en el desarrollo de la autoinmunidad.

En el **estudio IV** investigamos los niveles de Inmunoglobulinas y autoanticuerpos en saliva. Este es un estudio piloto y hasta ahora, las medidas de anticuerpos en saliva reflejan los perfiles serológicos en LES, con y sin SS. Nuestro objetivo a largo plazo es evaluar si los autoanticuerpos en saliva pueden ser usados para el diagnóstico, monitoreo y predicción de SS en LES u otras características autoinmunes.

En concreto, esta tesis demuestra el potencial de la cavidad oral para proveernos de indicadores biológicos y como estos indicadores pueden contribuir a la identificación de vías hacia la autoinmunidad. Éstos novedosos hallazgos podrían facilitar la detección temprana de estas enfermedades autoinmunes, y evitar daño acumulado en los órganos inducido por LES y otras condiciones autoinmunes.

Abstract

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease with a 9:1 female predominance where many genetic and clinical characteristics overlap with other inflammatory conditions such as rheumatoid arthritis (RA). Closely monitoring and treating active disease leads to better prognosis and quality of life for these patients since a cure is to date not available. In this thesis, we explored the oral cavity and its potential to offer markers of systemic inflammation as well as its possible role in explaining pathophysiological mechanisms behind autoimmunity.

In **study I,** we demonstrated that Sjögrens syndrome (SS) affects *1/4* of SLE patients, a higher prevalence than earlier reported (6-14%), which strengthens the importance of the oral cavity and the salivary glands in SLE. The SLE subsets with vs. without SS were compared clinically and immunologically, and we identified that potential markers of systemic inflammation were higher in the SLE with secondary SS group.

In **study II**, we compared potential biomarkers for disease activity (DA) in parallel samples of serum, saliva, and urine from SLE patients. Colony-stimulating factor -1, tumor necrosis factor-α, interferon-γ-induced protein -10, and monocyte chemoattractant protein -1 as well as calprotectin in saliva, discriminated SLE patients from controls and were associated with higher DA. Our findings demonstrate the potential of saliva as an alternative provider of biomarkers often with similar results as serum and urine.

In **study III**, we investigated the association of autoantibodies and antibodies towards *Pg* virulence factor arginine gingipain (Rgp) IgG with the occurrence and severity of periodontitis (PD) in three well-characterized study populations of SLE and PD patients. Anticitrullinated protein antibodies (ACPA) associated with the occurrence of PD, and ACPA and dsDNA antibodies associated with arginine gingipain (Rgp) IgG antibodies, which also associated with PD severity. The association of these autoantibodies with PD supports the rationale for further studies of oral pathways to autoimmunity.

In **study IV,** we investigated Immunoglobulin and autoantibody levels in saliva. This is a pilot study and this far, the antibody measurements in saliva reflect the serum profiles in SLE with and without SS. Our long-term goal is to evaluate if salivary autoantibodies can be used to diagnose, monitor or predict SS or other autoimmune features in SLE.

Overall, the studies in this thesis demonstrate that the oral cavity provides us with alternatives to explore biomarkers. These insights may help to identify novel pathways to autoimmunity. Our novel observations may also contribute to simplified testing and monitoring of DA, and possibly to earlier detection and treatment, to prevent organ damage in SLE and other autoimmune conditions.

List of scientific papers

I. **Guillermo Ruacho***, Marika Kvarnström*, Agneta Zickert, Vilija Oke, Johan Rönnelid, Susanna Eketjäll, Kerstin Elvin, Iva Gunnarsson, Elisabet Svenungsson
Sjögren´s syndrome in Systemic Lupus Erythematosus- a subset characterized by a systemic inflammatory state
The Journal of Rheumatology, 2019, Vol. 49, issue 10

* MK and GR contributed equally as first authors

II. **Guillermo Ruacho**, Ronaldo Lira-Junior, Iva Gunnarsson, Elisabet Svenungsson, Elisabeth A Bostrom
Inflammatory markers in saliva and urine reflect disease activity in patients with systemic lupus erythematosus
Lupus Science & Medicine, 2022, Vol. 9, issue 1

III. Charlotte De Vries*, **Guillermo Ruacho***, Elin Kindstedt, Barbara Aleksandra Potempa, Jan S. Potempa, Björn Klinge, Pernilla Lundberg, Elisabet Svenungsson and Karin Lundberg
Antibodies to Porphyromonas gingivalis Are increased in Patients with Severe Periodontitis, and Associate with Presence of Specific Autoantibodies and Myocardial Infarction
Journal of Clinical Medicine, 2021, Vol. 11, issue 4

* CDV and GR contributed equally as first authors

IV. **Guillermo Ruacho**, Ronaldo Lira-Junior, Marika Kvarnström, Agneta Zickert, Vilija Oke, Johan Rönnelid, Iva Gunnarsson, Elisabeth Bostrom and Elisabet Svenungsson
Autoantibodies in saliva in patients with systemic lupus erythematosus

Manuscript

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List of abbreviations

Aa	*Aggregatibacter actynomicitemcomitans*
ACPA	Anticitrulinated protein antibodies
ACR	American College of Rheumatology
ANA	Antinuclear antibodies
AUC	Area under the curve
AECC	American-European Consensus Criteria
AUROC	Receiver-operating characteristic curves
APC	Antigen presenter cells
aPL	Antiphospholipid antibodies
BOP	Bleeding on probing
BILAG	British Isles Lupus Assessment Group
CI	Confidence intervals
CL	Cardiolipin
CAL	Clinical attachment loss
CCP	Cyclic citrullinated peptide
CSF	Colony stimulating factor
CVD	Cardiovascular disease
DA	Disease activity
DC	Dendritic cell
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
Fc	Fragment crystallizable
GC	Germinal center
GP	Glycoprotein
GCF	Gingivocrevicular fluid
HIV	Human immunodeficiency viruses
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IFN	Interferon

Ig	Immunoglobulins
IL	Interleukin
IP	Interferon-γ-induced protein
IQR	Interquartile range
LN	Lupus Nephritis
LPS	Lipopolysaccharides
MCP	Monocyte chemoattractant protein
MI	Myocardial infarction
MIP	Macrophage inflammatory protein
MSD	Mesoscale discovery
NETs	Neutrophil extracellular traps
OC	Oral cavity
OM	Oral mucosa
OMB	Oral microbiome
PD	Periodontal disease or periodontitis
Pg	*Porphyromonas gingivalis*
PAD	Peptidyl arginine deiminase
PDC	Plasmacytoid dendritic cells
PMN	Polymorphonuclear
PPD	Probing pocket depth
PRR	Pattern recognition receptors
pSS	Primary sjögrens syndrome
RA	Rheumatoid arthritis
Rgp	Arginine gingipain
RF	Rheumatoid factor
SD	Standard deviation
Sm	Smith
RNP	Ribonucleoprotein
SS	Sjögrens syndrome
SAP	Seropositive-arthralgia patients
SE	Shared epitope

SLE	Systemic lupus erythematosus
SPSS	Statistical package for social sciences
SLAM	Systemic lupus activity measure
SLICC	The SLE International Collaborating Clinics
SLEDAI	Systemic lupus erythematosus disease activity
SLEDAI-2K	The SLEDAI 2000
Socialstyrelsen	The National Board of Health and Welfare
sSS	Secondary Sjögrens syndrome
SSA/Ro	Antibodies to Sjögrens syndrome, antigen A
SSB/La	Antibodies to Sjögrens syndrome, antigen B
TGF	Transforming growth factor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
WS	Whole-saliva
WUSF	Whole unstimulated salivary flow

1 Introduction

1.1 Systemic autoimmune diseases

Systemic autoimmune diseases are a group of diseases that have many clinical features in common and partly overlap one another regarding symptoms, autoantibodies, and risk genes. These diseases include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Sjögrens syndrome (SS), and several less common conditions. The causes of these diseases remain unknown but the importance of autoantibodies; such as rheumatoid factor (RF), anticitrulinated protein antibodies (ACPA), and antinuclear antibodies (ANA) as well as several ANA subspecificities has been suggested in the pathogenesis. However, these autoantibodies can also be present in healthy individuals and commonly they appear before disease onset [1,2].

1.1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune systemic disease characterized by enhanced autoantibody production and the formation of immune complexes. SLE is a heterogeneous condition involving several organ systems and disease activity varies from persistently mild to life-threatening [3]. A state of systemic inflammation associated with enhanced activity in the type-1 interferon (IFN) system, and high levels of pro-inflammatory cytokines, e.g. Tumor necrosis factor (TNF)-α, Interleukin (IL)-6, IL-8, IL-16 and interferon-γ-induced protein (IP)-10 is common in SLE [4-7] **(Figure 1).**

SLE has a gender predilection where 9 out of 10 patients are women. In studies from 1988 differences in the prevalence regarding different ethnicities were reported [8]. In a similar but more recent worldwide study of SLE prevalence, figures as high as 241 cases per 100 000 were reported in North America [9]. The high frequency of SLE reported in the latter study might have been due to ethnicity influence of the African American population, in which it is well known that the prevalence is higher. The survival rate in SLE has also changed over the years; in an older study from 1955, a 5-year survival of 51% was reported [10], whereas, in a systematic review from 2017 the 5-year survival rates exceed 95 % [11].

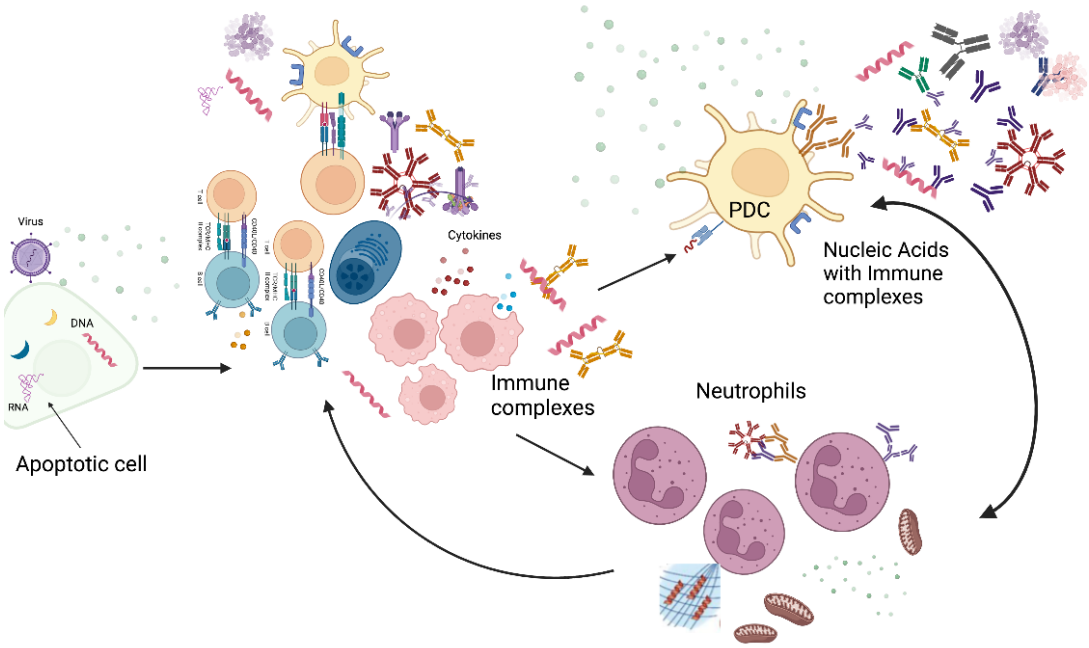


Figure 1. The type I interferon system in SLE and SS. Schematic representation of the activation of the type I IFN system. The innate immune responses in SLE and SS are linked with apoptotic clearances, consequent break of tolerance, and an enhancement of type I IFN system mainly linked to IFN production by plasmacytoid dendritic cells (PDC). A dying cell expels its content – increase shift in CD-4 APC – interaction bring self-autoantigens to lymph-node, the T cells present to B cellS – produce autoantibody – that circulate back to the tissue and create immune complexes. IFN may be activated due to the presence of free nucleic acids (dead cells) or by deposited immune complexes. In SLE the PDCs are stimulated by the extracellular traps from the NETosis of Neutrophils. A systemic inflammation starts and high levels of proinflammatory cytokines occur [4,12,13]. Illustration created in Biorender.

SLE is a clinically diagnosed condition, but diagnostic criteria are still lacking. SLE can affect many organs including joints, skin, mucosa, and the kidneys. The severity of SLE depends on the extent of accrued damage and disease activity (DA), which varies from persistently mild to complications such as lupus nephritis (LN) [3] or severe neurological involvement. Biopsy-based tests can help in diagnosis [14] but for research purposes, SLE has been defined by different sets of clinical and laboratory classification criteria.

1.1.1.1 SLE classification criteria

Several sets of criteria have been created to classify SLE but the most known and accepted worldwide are the 1982 American College of Rheumatology (ACR) revised classification criteria [15].These criteria have, over the years, been modified and adapted according to the advances in the understanding of the disease [15-18]. There are two more recent attempts to create a validated set of SLE classification criteria; the SLE International Collaborating Clinics (SLICC) group published one set in 2012 [19], and the collaboration between the ACR and the European League Against Rheumatism (EULAR) published another set in 2019 [20]. Variations in criteria used for research purposes may also substantially influence the prevalence reported from the different epidemiological studies.

Table 1. The 1982 revised criteria for SLE classification

Criterion	Definition
Malar rash	Erythema over the malar eminences
Discoid rash	Erythematous raised patches
Photosensitivity	Skin rash due to unusual reaction to sunlight
Oral ulcers	Oral or nasopharyngeal ulceration
Arthritis	Non-erosive arthritis involving two or more peripheral joints
Serositis	Pleuritis or Pericarditis
Renal disorder	Persistent proteinuria >0.5 g/day or cellular casts
Neurologic disorder	Seizures or psychosis
Hematologic disorder	Hemolytic anemia, leukopenia, lymphopenia, or thrombocytopenia
Immunologic disorder	anti-DNA, anti-Sm or a false positive serologic test for syphilis
Antinuclear antibody	Abnormal titer of ANA

The 1982 ACR revised classification criteria are based on 11 criteria; 9 clinical and 2 immunological. If any of 4 or more of the 11 criteria are present, the individual is classified as having SLE. Adapted from Tan EM [15]

1.1.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory autoimmune condition characterized by symmetrically affected synovial joints, with a prevalence from 0.5 to 1% worldwide, and a female-to-male ratio of around 3:1 [21,22]. RF and ACPA are present in a majority of patients (around 70%) and define the *seropositive* subset of RA, characterized by a more severe and destructive disease course [23]. RFs bind to the constant fragment crystallizable (Fc) region of immunoglobulins of the IgG isotype, while ACPA are directed towards proteins that have been post-translationally modified by citrullination, a process where the peptidyl arginine deiminase (PAD) enzyme transforms peptidyl-arginine into peptidyl-citrulline [24-26]. Both RF and ACPA are part of the classification criteria for RA [24] and are important for diagnosing patients. RF can be found in other conditions, while ACPA are highly specific for RA.

The heritability of RA is currently estimated to be 40–65% for seropositive disease, but lower (20%) for seronegative disease [26]. Recent studies have demonstrated that autoimmune diseases can develop in genetically predisposed individuals when exposed to specific environmental factors, triggering immune responses. In RA, carriage of the *HLA-DRB1* "shared epitope" (SE) genes and smoking cigarettes are two independent risk factors, which in combination increase the risk of ACPA-positive RA. Interestingly, these two risk factors have also been associated with the occurrence of antiphospholipid antibodies (aPL) [25,27,28]. Moreover, *HLA-DRB1* SE and smoking have also been shown to associate with periodontal disease (PD) [27], and aPL with cardiovascular disease (CVD) [29], though so far only in studies of limited size.

1.1.3 Sjögrens syndrome

Sjögrens syndrome (SS) is a systemic multifactorial inflammatory autoimmune condition that affects genetically susceptible individuals [30]. The diagnosis SS is a clinical entity, based on *sicca symptoms;* dryness of eyes (xerophthalmia) and mouth (xerostomia) due to destructive inflammation in the exocrine glands, especially tear and salivary glands. The estimated prevalence is 0,5% and 9 out of 10 patients are female [31,32].

Activated type I IFN genes, often referred to as the IFN signature; are a hallmark of SS. Toll-like receptors (TLRs) and B-lymphocytes also play an important role in the immunopathogenesis of SS. Focal infiltration of mononuclear cells in exocrine glands is the main histopathological characteristic in SS, but what initiates this infiltration and colonization of mononuclear cells remains to be elucidated. A deficiency in the apoptosis of epithelial cells of exocrine glands might be the cause of intracellular proteins becoming autoantigens. Consequently, loss of tolerance occurs and plasmacytoid dendritic cells (PDC) enhance the production of type I, II and III IFN and antigen presentation attracting T-lymphocytes and consequently activating B-cell production of autoantibodies [33 34] **(Figure 1)**.
The excess production of Immunoglobulins (Ig) results in hypergammaglobulinemia, and local production of antibodies to Sjögrens syndrome A and B antigens (SSA/SSB, also referred to as Ro/La) occurs in the germinal center- (GC) like structures within the target tissue [35,36].

Accumulating data suggest that oral "mucosal breaks" can drive the initiation of systemic diseases [37], whether these breaks are driven by microbes consequently triggering autoimmunity has also been suggested [38]. Extravasation has also been studied in SS and proteomic studies in saliva attempt to explain the oral origin of SS [39,40]. The possibility that the autoantibodies in SS are locally synthesized and secreted within the salivary glands and that B cells proliferate clonally in the salivary glands [41], was proposed years ago [42]. These studies reported that IgA anti-La antibodies were detected in saliva in several cases when IgG anti-La antibodies in serum was still negative, suggesting that IgA class autoantibodies can be detected in saliva before they appear in the circulation.

SS can exist isolated and is called primary (p)SS, or together with other inflammatory rheumatic diseases, referred to as secondary (s)SS. The most frequent autoantibodies in SS are ANA anti-SSA/Ro followed by SSB/La and RF. A major difference according to the 2002 Revised American-European Consensus Criteria (AECC) is the classification, where the serologic item (SSA/SSB antibodies) is included for pSS, but not for sSS [43,44] **(Tabel 2 and 3).**

1.1.4 Secondary-Sjögrens syndrome

The existence of different subsets of the SLE population was already suggested in 1959 by Heaton et al. describing the subset with SS as a chronic and relatively benign form of SLE [45]. More recently, several studies have identified autoantibody clusters/immune phenotypes, which vary concerning clinical symptoms, biomarkers, genetic susceptibility, and prognosis [46-51]. A SLE phenotype characterized by antibodies to SSA/Ro and SSB/La, consistently appears in these studies.

In SLE, SSA/Ro and SSB/La autoantibodies are common, usually stable over time and they appear early, even several years before disease onset [1,52-54]. The clinical SLE-sSS phenotype has been presented in the literature with a dominance of skin and joint manifestations and less severe internal organ involvement, especially less nephritis. The occurrence of SS in SLE has in most previous studies been reported to be between 6 % and -14 % [55-59]. However, this supposedly milder SLE subset has so far achieved limited scientific attention [45,55,60]. Differences and similarities between pSS and SLE-sSS have been studied [56,61], but to what extent the inflammatory pattern differs between SLE-sSS and SLE patients without SS is not known, and this information may be important concerning treatment perspectives.

High susceptibility for manifestations in the oral cavity (OC) is known to be associated with immunodeficiency conditions, involving the reduction of the salivary flow and ulcers in the oral mucosa [62]. Patients with conditions such as SLE may have aphthous ulceration episodes and oral ulcers are one of the criteria included in the three major classification criteria for SLE [15,19,20].

Table 2. Autoantibodies in autoimmune diseases

Autoantigen	Association	Specificity	Frequency % ([63-65])	Frequency % KS
ANA	Autoimmune disease in general, also highly present in the healthy population	Low- SLE, SS, RA	95	NC
Anti-dsDNA	Nephritis, skin-and cerebral lupus	High SLE specific	60-90	39
Anti-Nucleosome	LN	More than anti-dsDNA but has detection problems	50-90	NC
Anti-Sm	LN	High in SLE	20-40	19
Anti-RNP	Raynauds phenomenon	IgM in isotype SLE, IgG isotype Mixed connective tissue disease (MCTD)	20-30	26
aPL	Antiphospholipid syndrome (APS)	Unspecific	30-40	NC
Anti-C1q	Complement pathways	Up to 100 % of active proliferative LN patients	20-50	NC
Anti-Ribosomal	Neuropsychiatric Lupus and LN	High in SLE	10-40	NC
Anti-SSA/Ro52	SICCA symptoms and SS	SLE, SS	30-40	28
Anti-SSA/Ro60	SICCA symptoms, SS, and neonatal Lupus	SLE, SS	30-40	41
Anti-SSB/La	SICCA symptoms, SS, and neonatal Lupus	SLE, SS	10-15	23
RF	RA, SS	IgA: SS and RA IgM: RA, DA in SS IgG: RA	60–80 in RA	32 24 15

Table 2. Presentation of autoantibodies and their distinct disease association. Comparison of frequencies based on our cohort and Maslinska et al., Dema et al., and Xiao et al. NC= not calculated

1.1.4.1 SS classification criteria

The criteria used in **study I** and **study IV** for the classification of sSS is the 2002 AECC, presently used worldwide. Conversely to the SLE criteria, the AECC criteria have been even used for diagnosis due to their high specificity and sensitivity, close to 100% [44]. Nonetheless, it has not been validated for the latter purpose. There are numerous criteria for both classification and diagnosis of SS, and for clinical trials [66]. In Sweden though, according to *Socialstyrelsen* (the National Board of Health and Welfare), the diagnosis should be based on the AECC.

Table 3. The 2002 AECC. Classification criteria for secondary-Sjögrens syndrome

Symptom	Type of measurement	Item	Instrument
Dry eyes	Subjective	I.	Questionnaire
	Objective	III.	- Schirmer´s test: ≤ 5 mm/ 5 min - Other
Dry mouth	Subjective	II.	Questionnaire
	Objective	V.	- Unstimulated whole saliva flow: ≤ 0.1 mL/min - Other
Lip biopsy	Objective	IV.	Labial salivary gland biopsy: Focus score ≥1

Item VI (serology) not included in the table as is not included for classification of secondary-sjögrens syndrome. Adapted from American-European Consensus Group 2002 [44].

The 2002 AECC classification criteria include oral and ocular symptoms, as well as histopathologic and serologic measurements. To be classified as having pSS, the serology (VI) or the histopathology (IV) item must be fulfilled in combination with the presence of four of all the items or three of the objective ones. To be classified as having sSS, another rheumatic disease must be present in combination with subjective symptoms reported, and at least 2/3 of the following items: Schirmer test (III), whole unstimulated salivary flow (WUSF) (V), or biopsy positivity with focus score ≥1 (IV) must be present.

Interestingly, for the classification of sSS, the item (VI) for the serology is not included.

1.1.4.2 Lip biopsies

Lip biopsies to obtain labial salivary glands is the histopathology item (IV) in the classification and diagnosis of SS [44]. Lip biopsies may also serve as a biomarker per se [67].

To define a lip biopsy as positive, focal lymphocytic sialadenitis is assessed. The presence of aggregates of fifty lymphocytes per 4mm^2 calculated as focus score ≥1, is the standard in the 2002 AECC.

Lip biopsies are performed as routine praxis for diagnosis of SS at the Department of Rheumatology at the Karolinska University Hospital in Stockholm County, Sweden.

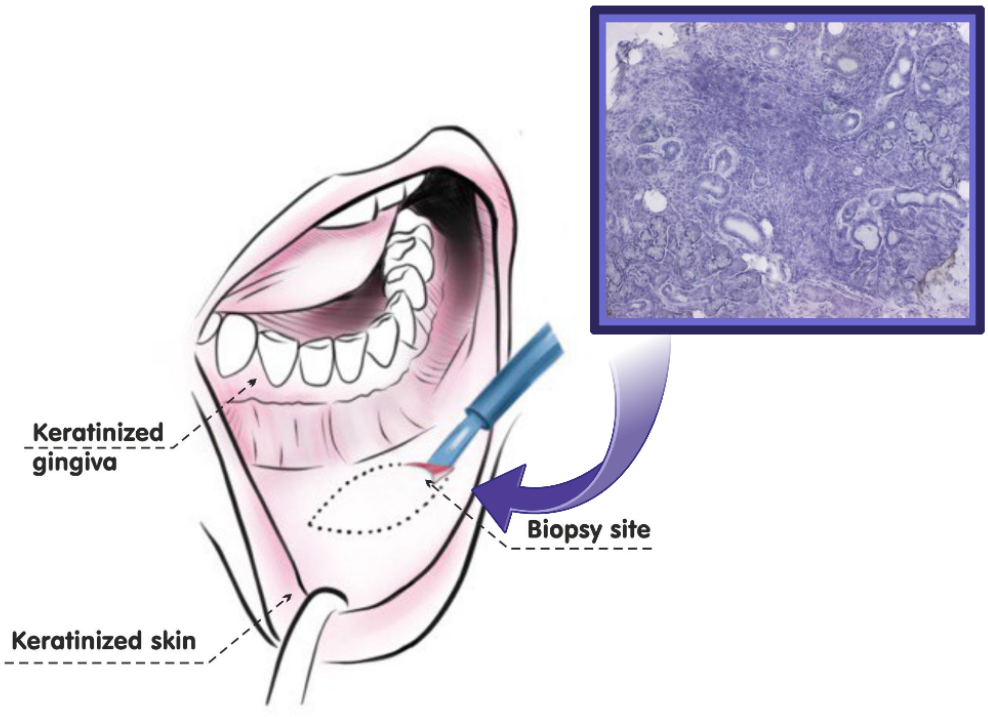


Figure 2. Lip biopsies. Cartoon, illustrating the site of biopsy and a positive focus score ≥1 per 4mm. By Guillermo Ruacho, pathological image courtesy of Marika Kvarnström.

1.2 Cardiovascular diseases

Cardiovascular disease (CVD) is a leading cause of mortality; in Europe it contributes to almost half of all deaths/year, and roughly one third of CVD fatalities are in individuals younger than 65 years old [68]. Subclinical CVD is also referred to as atherosclerotic disease or atherosclerosis, which indicates a chronic inflammation and the formation of plaques in the arterial walls and may evolve into myocardial infarction (MI) [69,70]. The pathogenesis of atherosclerosis is linked to traditional CVD risk factors such as age, gender, hypercholesterolemia, hypertension, diabetes, and smoking.

Low-grade chronic inflammation is associated with the development of atherosclerosis, and it is known that chronic inflammatory conditions like RA and SLE can accelerate its progression and inflammatory activation increases the risk for plaque rupture leading to acute coronary syndromes [71,72]. PD is another chronic inflammatory condition, that affects the supporting structures around the teeth, but it also seems to be a low-grade systemic inflammation in patients with severe PD. Recently, a study from our group showed that PD associates with first MIs [73].

Cardiovascular events represent a growing share of mortality causes in SLE [69,74,75] and aPL has been demonstrated to serve as predictors for the risk of cardiovascular mortality in SLE patients [69]. aPL is a group of antibodies that recognize phospholipids and proteins which are part of or bind to membrane structures. The presence of aPL increases the risk for thrombosis and aPL are common in patients with SLE, although they also occur in the general population, where they have been much less studied [76,77] **(Table 2).** Moreover, in our group, we have previously reported significantly higher levels of aPL in MI *vs.* healthy controls [29] in the *PAROKRANK* group.

1.3 The oral cavity and oral mucosa

The oral cavity (OC) is the first portion of the gastrointestinal tract where the mucous membrane is a prolongation of the intestinal lining from the pharynx. In homeostasis, the healthy OC is a moist environment where the oral mucosa (OM), with the help of saliva, conforms to a barrier against pathogens. The OM has two separate tissue components: a covering epithelium and underlying connective tissue, which both take part in the major functions of the OM, i.e., lining and protecting. The covering mucosa is flexible which is important for its protective function and when it surrounds an erupted tooth is known as gingiva and forms part of the tooth periodontium.

The junctional epithelium that attaches the gingiva to the tooth is permeable and antigens can pass through and start immune responses and inflammation in the gingival tissue. The importance of the innate immunity and the local cells has been highlighted: host detection of microbes happens through pattern recognition receptors, including TLRs, expressed on these cells [78].

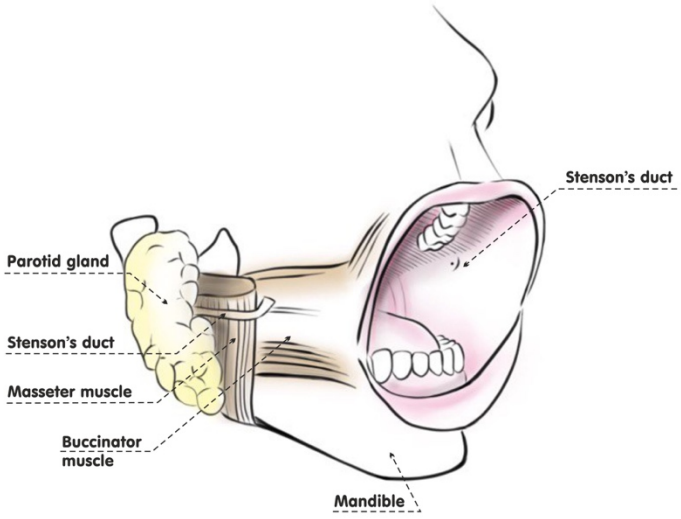


Fig 3. Anatomical relations of the oral cavity. Illustration showing the salivary glands and their secretory ducts in the oral mucosa. By Guillermo Ruacho

The OC has often been studied as an isolated single entity, sometimes with a few attempts, as with the *oral focal infection* theory, to present it as the origin of infections in the rest of the body [79,80]. The OC has, therefore, controversially been disregarded when exploring solutions to systemic health. Fortunately, this view is now changing and an interest in the involvement of the OC and the composition of saliva has gained increased attention [81,82].

1.3.1 The oral mucosa mirror of systemic diseases

The OC has been suggested to herald initial clinical signs of several diseases such as SS, SLE, RA, and CVD [83-88]. Similarly, some studies imply that oral lesions can announce gastrointestinal symptoms in inflammatory bowel disease (IBD) [89,90]. These findings indicate a potential benefit for the understanding of systemic conditions by studying the involvement of the OC in several diseases.

1.3.2 Saliva and salivary glands

Saliva is a mucoserous secretion **(Figures 4 and 5)** produced by the exocrine salivary glands [82] and whole-saliva (WS) is a compound fluid, which contains other non-exocrine components **(Figure 4)**. The different salivary glands contribute to the WS in different percentages: 20% from parotid, 65% from submandibular, 7% to 8% from sublingual, and less than 10% from numerous minor glands in unstimulated flow. During stimulation, the proportions change considerably, and the parotid secretion represents more than 50 % of the whole secretion [81,91]. In homeostasis, the daily flow of saliva varies between 1 and 1.5 L in healthy individuals.

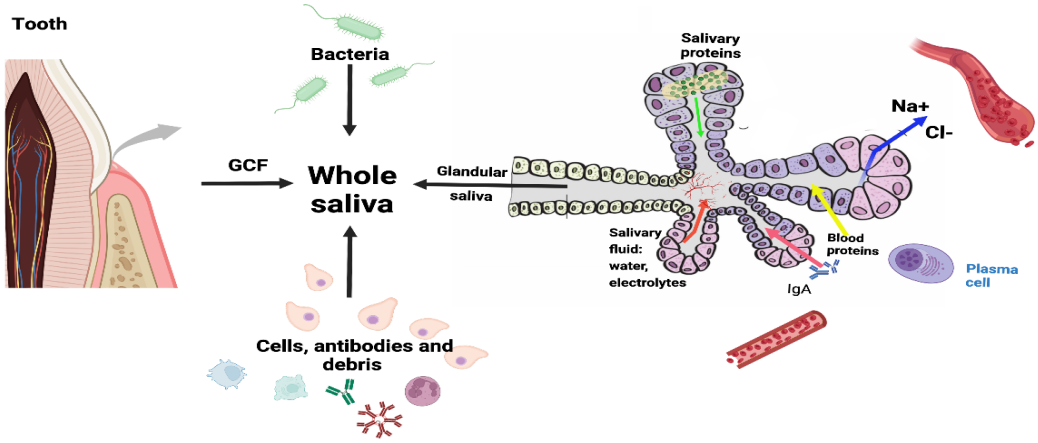


Fig 4. Whole saliva. WS is a compound fluid, mainly formed from major and minor salivary glands secretions but it also contains the gingivocrevicular fluid (GCF); a serum transudate from the gingiva [92,93], bacteria, antibodies, and food debris [81,91,94]. Illustration created in Biorender.

1.3.2.1 Xerostomia or stomatitis sicca

Xerostomia and sicca symptoms are two Latin terms that refer to dryness of the oral cavity caused by insufficient or a complete lack of saliva secretion. The prevalence of xerostomia varies between 12 and 30% and its cause can be classified as local or systemic. Local factors responsible for mouth dryness include conditions or damage in the salivary glands such as sialadenitis, sialolithiasis as well as radiotherapy, or infections or neoplasms in the oral cavity, pharynx, or in oesophagus [95,96]. Among the systemic causes of xerostomia, we find Alzheimer´s disease, Parkinson´s syndrome, and human immunodeficiency viruses (HIV) infection. Additionally, the salivary glands are targets in systemic diseases such as SS, SLE, and RA. SS is thought to be the cause of stomatitis sicca in these other conditions. There is ongoing debate as to whether SS together with other diseases are two overlapping conditions or if SS is a secondary condition to these diseases [56,57].

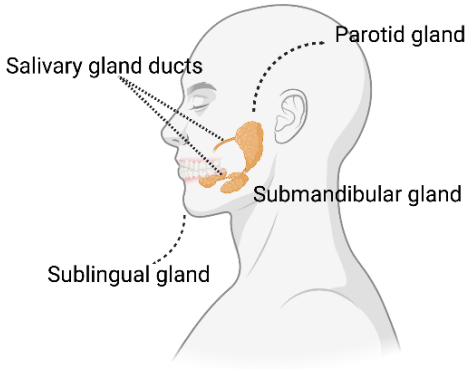


Fig 5. Salivary glands. There are three major pairs, which differ in their type of secretion: the parotid (serous), submandibular (mixed), and sublingual (mucous/mixed). They secrete their products into the OC via extended duct systems. Illustration created in Biorender.

1.3.2.2 Saliva as a mirror of systemic status

Changes in the salivary microbiome (MB) with the presence of systemic conditions have been studied [97] and the analysis of salivary proteins has been thought to mirror the status of the salivary glands, the target organs in SS [98].

SLE has often been referred to as the prototype of a multisystemic inflammatory condition. Salivary levels of inflammatory cytokines have also been shown to be elevated in patients with SLE [99,100]. These findings imply that saliva could be a potential tool to diagnose and monitor autoimmune diseases. In general, body fluids other than blood have the advantage to identify markers of systemic status, by being collected without invasive procedures.

1.3.2.3 Protein concentrations in saliva

Different factors may influence the salivary proteome profile. As saliva samples have the potential to be used for diagnostic or monitoring purposes, it is of great interest to characterize these factors. It is presumed that 0.3 % of the total saliva consists of proteins and among these the main components are enzymes, glycoproteins, immunoglobulins, and antimicrobial peptides [101]. The composition of saliva may vary, partly because these peptides are susceptible to hydrolysis by proteolytic enzymes from oral bacteria, and this needs to be taken into consideration when working with saliva.

Loo et al. compared proteins in saliva and plasma and found that approximately 1/3 of the WS proteins are found in plasma and that almost 1/2 of the proteins which are currently suggested as disease biomarkers for diseases such as CVD, among others are found in WS [102]. Additionally, a comparison of the proteomic profile of saliva from individuals with healthy or bleeding oral cavities showed that some proteins increased two- fold or more in individuals with bleeding [103]. Furthermore, comparisons of the proteome of whole and glandular saliva with different methods of collection showed considerable differences in both the morphology and morphometry of the salivary proteome depending on the sampling methods [104] i.e. T-helper 2 proteins associated with hyposalivation [105]. Taken together, these findings stress the importance of consistency when collecting saliva for proteomic analysis.

1.4 The oral cavity and oral microbiome

The study of the human microbiome is a constantly evolving field. In recent years, novel concepts have emerged to explain host-microbial interactions [106,107]. Accordingly, the study of the oral microbiome (OMB) has parallelly evolved and applied the gained knowledge [108,109]. For a long time, it was thought that affections of the OC, such as periodontal disease, or dental caries were caused by an overgrowth of single pathogens. However, it has been shown that the OC is colonized by a variety of microbial species, including those found in the healthy OMB. This change of paradigm suggests that these common affections are rather related to a dysbiosis of the OMB and its influence on the salivary composition.

Recently, Gardner et al. [110] studied the influence of the OMB on salivary metabolic composition. These results confirmed the contribution of the OMB to the salivary metabolome but also demonstrated that metabolites such as lactate, urea, and citrate present in WS originate from the host circulation. Additionally, this study demonstrated that similar metabolic profiles are present in plasma, parotid saliva, and WS [110]. Finally, certain bacteria from the OMB and the salivary glands have proved to contribute to nitric oxide homeostasis by metabolizing nitrate [111]. The effect of this has been presumed to have implications not only locally in a probiotic manner but also at a systemic level, as a blood pressure regulator [112].

Other studies have focused on the interaction among bacteria, particularly their symbiosis/cohabitation, by studying their metabolites. Zhu et al. showed that *Aggregatibacter actynomicitemcomitans* (*Aa*) plays an important role in the undisturbed adhesion of the keystone pathogen *Porphyromonas gingivalis (Pg)* [113] to the biofilm, protecting from other bacterial metabolites that may negatively influence its growth [114].

Taken together, these studies agree that certain metabolites present in WS originate from the host circulation, but the products of the colonizing bacteria also account for a significant portion. Therefore, in cases when sterile saliva obtained from the parotid gland lacks certain metabolites it is because it is sterile, and has not yet been contaminated by proteins of bacterial origin as suggested by Jasim et al. who also emphasize the importance of consistency when collecting saliva [104].

1.4.1 Dysbiosis of the oral microbiome and the immune system

Interactions between the immune system and the oral microbiome have been reported in many studies. Dzunkova et al. suggest that this interaction per se causes oxidative stress, which at the same time results in temporal variations of the oral microbiome [115]. Other studies demonstrate that oral dysbiosis and a high number of periodontal pathogens are present in SLE patients and that the presence of antibodies against bacteria such as *Aa, Pg, Treponema denticola,* and *Capnocytophaga ochracea* correlates with higher levels of anti-dsDNA and reduced levels of complement proteins [116-118]. Nonetheless, a recent report on patients with pSS suggested that a reduced salivary secretion has a stronger influence on the oral microbiome than the disease per se [119]. However, in this study they focused on bacteria more related to caries such as *Rothia dentocariosa, Veillonela sp., Prevotella salivae, Streptococcus mutans,* and *Lactobacillus* [120]. It would be of interest to know the caries activity of these patients since xerostomia favors caries and consequently the colonization of these bacteria.

It has been proposed that one of the causes of flares in systemic diseases, such as SLE, may be exposure to infections, which can trigger the activation of both the innate and adaptive immunity through the co-stimulation occurring during antigen presentation, stimulated by pathogen-associated molecular patterns [121]. Similarly, a potential role of the *Epstein-Barr virus* (EBV) in SLE [122], SS [38], and more recently in RA [123], has been proposed. The importance of EBV has however been subject to many studies and its role in these diseases is considered controversial. Some studies suggest an immune response against the Epstein-Barr nuclear antigen-1 and imply that an association with specific antibody epitopes exists [124,125].

1.4.2 Dysbiosis of the oral microbiome and periodontitis

Periodontitis or Periodontal Disease (PD) is an inflammatory disease that affects the supporting structures around the teeth (the gingiva, bone, and periodontal ligament) and may lead to tooth loss in susceptible individuals [113]. In the Western European population, the prevalence of severe PD is approximately 9% [126], whereas, in The United States, an age-dependent increase of the prevalence from 11% to 20% has been reported among older age groups. In children and youth, the occurrence of PD is lower than in older individuals [127].

Throughout the years the definition of PD has been clinical, using parameters such as bleeding on probing (BOP), probing pocket depth (PPD), clinical attachment loss (CAL), and radiographic bone loss to determine the pocket depth. Percentages of teeth presenting pockets or CAL above specific measures are present in the definition. Consequently, this definition specifies certain forms of periodontitis from local to general and from mild to severe [128-130]. In contrast, a new classification system was presented more recently, classifying PD in stages (I-IV) defined by severity, complexity, and extent and distribution as well as by grades (A-C) as defined by evidence of, or risk for rapid progression, anticipated treatment response, and effects on systemic health [131].

PD is a chronic inflammatory condition, induced by dysbiosis in the OMB due to increased oral plaque and its posterior colonization of gram-negative- and anaerobic pathogens and pathobionts bacteria [113]. The microbial biofilm grows and expands to the periodontium and its interaction with the immune system of the host, leads to inflammation and PD [128]. The OMB contains aerobic and anaerobic bacteria that in health/ homeostasis live in symbiosis. The adherence of the oral biofilms to the dental surfaces is a complex environment for the bacteria where they, in interaction, succeed to grow independent colonies. More than 500 bacterial species can be found in a dental plaque [132], but by using new technology, it has been shown that a larger number of bacteria are present in the OC [133]. Among the colonizing bacteria in the periodontium, the following are closely related to PD: *Pg*, *Tanarela Forsynthya,* and *Treponema Denticola*, a group of bacteria earlier identified as *the red complex* [134].

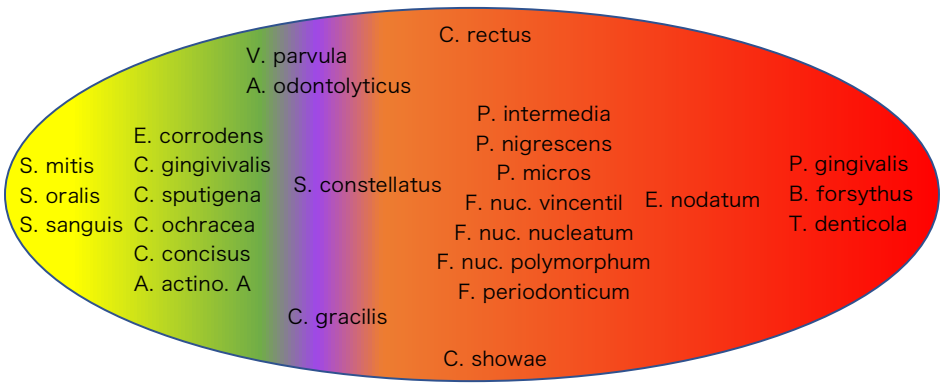


Figure 6. The red complex and Periodontitis. Relationships of microbial species and their classification within microbial complexes according to Socransky et al. [134]. Illustration adapted by Guillermo Ruacho.

Although the presence of these bacteria and their colonizing is crucial for the development of PD, this is not the only pre-requisite for PD pathogenesis. Bacterial products such as lipopolysaccharides (LPS) induce the secretion of inflammatory mediators by binding to TLRs on host cells [135,136]. TLRs make up the prototypical and best-characterized pattern recognition receptor family (PRR) [137]. Gingival fibroblasts endure LPS through TLRs [138,139] however, something like a break of tolerance occurs when the host is unable to keep the balance in response to bacteria dysbiosis and reacts with an immune overreaction. A state of inflammation guided by bacterial metabolites acting as chemotactic gradients and causing infiltration of neutrophils and granulocytes starts. These inflammatory cells produce pro-inflammatory cytokines such as TNF-α, IL-1, IL-8, IL-12, IFN-γ, and transforming growth factor (TGF) -β as well as antibodies raised against the biofilm components. The inflammatory process will be followed by lymphocyte infiltration that interacts with dendritic cell (DC) antigen presentation. At this stage, tissue damage occurs because of the inflammatory process [128].

1.4.2.1 Periodontitis as a trigger of autoimmunity

The relation between the oral cavity and arthritis was already suggested by Hippocrates when he proposed that arthritis could be cured by pulling out bad teeth [140]. However, the underlying molecular disease mechanisms were not explained, but this is something that more recent studies have focused on.

It has been suggested that infections caused by bacteria trigger an autoimmune response and are involved in autoimmune pathogenesis. Periodontitis is initiated and maintained by the disturbance of the microbiome, but genetic and environmental factors also contribute to the appearance and progression of the disease. The innate immune system is very important in the initial phase of PD. Both polymorphonuclear (PMN) and mononuclear myeloid cells are important players during the initial lesion of the disease. Once the neutrophils accomplish their function they will undergo apoptosis to consequently be engulfed by macrophages, and lymphocytes will be attracted to the site of inflammation to further secrete more cytokines through co-stimulation [141]. Also, pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 are involved in the pathogenesis of PD [142]. Moreover, increased levels of IL-8 have for example been demonstrated in GCF from patients with PD with decreased IL-8 levels after periodontal therapy [143].

Similarly, the role of these cytokines has been highlighted in RA, with the successful use of cytokine-blocking treatments. Interestingly, RA patients affected with PD have also been reported to show reduced attachment loss after anti-TNF treatment [144]. The relationship between different cytokines and autoantibodies has been the focus of many RA studies during recent years. For example, it has been shown that ACPA induce IL-8 production by osteoclasts and that IL-8 blocking prevents ACPA-induced bone loss [145].

Chronic inflammation is involved in both PD, and autoimmune diseases, including SLE, RA, SS, and CVD. There is also an epidemiological association between PD and several of these conditions, *i.e.* RA, diabetes mellitus, and atherosclerosis [83-88,146]. Moreover, oral infections/PD have been suggested to directly contribute to the autoimmune reactions that characterize RA **(Figure 7).**

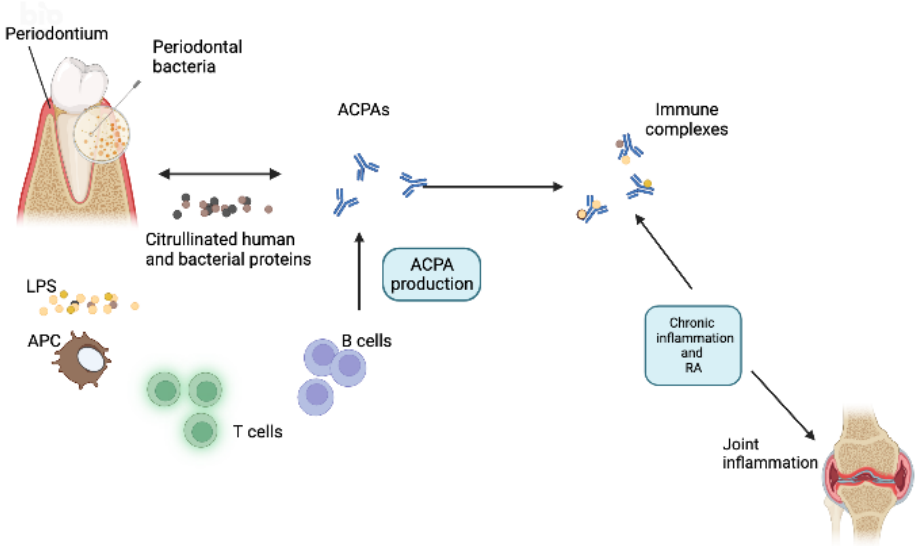


Figure 7. Periodontitis (PD) and autoimmunity, the rheumatoid arthritis (RA) connection. Illustration of the hypothesis behind the relationships of PD, citrullination, and ACPA production. The periodontal bacteria Porphyromonas gingivalis carry a PAD enzyme capable of citrullinating proteins, and another periodontal bacteria, Aggregatibacter actynomicitemcomitans, produce a toxin that can trigger activation of human citrullinating PAD enzymes and hypercitrullination in neutrophils, suggesting that these bacteria may be involved in the generation of citrullinated autoantigens, loss of tolerance and production of ACPA [27,147,148]. Illustration created in Biorender.

Of note, antibodies to *Pg* have been linked to ACPA-positive RA, even before the onset symptom [149-151]. However, conflicting data have also been presented, where some report no difference in anti-*Pg* IgG levels between ACPA-positive and ACPA-negative "seropositive-arthralgia patients", and only a weak correlation between anti-*Pg* IgG and ACPA was observed in individuals who developed RA [152]. Also, a more recent study concluded that anti-bacterial antibodies to other periodontal bacteria such as *Prevotella intermedia* [153] may indicate a potential role heralding RA.

Furthermore, as mentioned earlier, the best-known risk factors for RA, cigarette smoking, and *HLA-DRB1* SE, have also been linked to PD [27]. The *HLA-DRB1* SE alleles have specifically been associated with the erosive progression of the surrounding tissues of teeth in PD, and with the distinct joint erosion seen in RA. Marotte et al. even reported an association between *HLA-DRB1* SE and positive labial salivary gland biopsy/sicca symptoms, characterizing sSS in RA patients [154].

Taken together, accumulating data points towards a role for oral microbes/PD in the development of autoimmunity, although the mechanisms involved have not yet been elucidated.

1.5 Biomarkers for disease activity in autoimmune diseases

A variety of assessment tools is available for monitoring disease activity (DA) in SLE patients. These tools are based on a range of components, including general measures of immunologic and inflammatory status, specific monitoring methods of the organs and tissues that are involved as well as global assessments of DA both by physicians and patients. Recognizing DA is crucial and DA indices have become an important tool to monitor SLE patients to avoid life-threatening organ involvement. Although a better understanding of autoimmunity in SLE has been achieved, more objective evaluations of DA are still the goal to decrease the dependency on subjective measures [155].

According to the *National Institute of Health Biomarkers´ definition Working Group*, an ideal biomarker is highly sensitive, highly specific, clinically relevant, and non-invasive or as little invasive as possible [156]. Biomarkers may be biological, biochemical, and/ or molecular. The advent of techniques such as analyses of the proteome profile has opened access to identify new potential markers in complex diseases like SLE. Some clinical features of SLE are preceded by inflammatory cell infiltration which drives the production of pro-inflammatory cytokines and chemokines **(Figure 8).** How autoantibodies and the formation of immunocomplexes contribute to the initiation of these processes is still not clear.

In SLE some cytokines are upregulated and positively associated with DA [6]. Cytokines are immune-regulated signalling proteins that have previously been studied as potential markers for diagnosis, prognosis, and surveillance of DA. TNF-α, IL-6, IL-8, IL-10, IL-16 and type I–III IFN are cytokines that are commonly present in the blood of patients with SLE/SS/RA [4,6,100,157,158]**(Figure 1).**

Colony stimulating factor (CSF)-1 is a growth factor for myeloid cells that have been shown to induce monocytes to produce higher levels of TNF-α [159,160]. Previous studies of TNF-α in humans and CSF-1 in mice have shown their potential to discriminate between SLE patients and controls [6,160]. Enhanced IFN-γ and TNF-α production has been shown to stimulate IP-10 secretion [161]. Furthermore, earlier studies have reported higher levels of TNF-α and IP-10 in circulation and positive associations with DA in SLE and SS [6,162,163]. The monocyte chemoattractant protein (MCP)-1 expression is upregulated by TNF-α and is associated with SLE [164,165]. Another protein that has been found associated with DA in SLE is calprotectin, a molecule produced by monocytes [166,167] and can be synthetized by PDC, a cell population thought to be central in the pathogenesis of SLE [168] **(Figure 1)**.

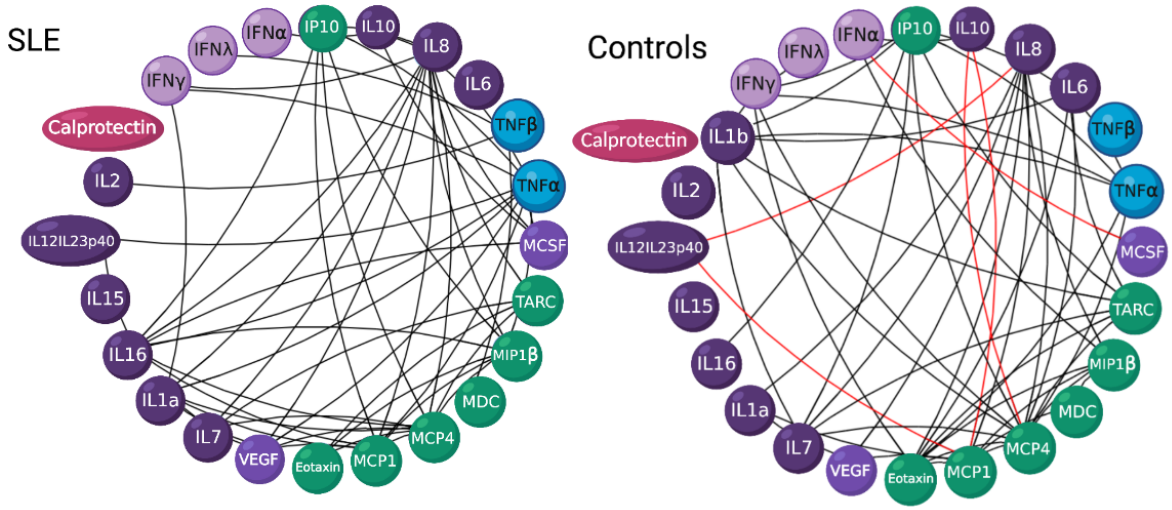


Figure 8. Cytokine network in serum from SLE patients and controls. Circulating cytokines and chemokines found in our SLE cohort, 6 out of 20 upregulated in the SLE-sSS subset. Network was built using significant correlation coefficients ($p<0.05$; $r\geq0.4$). The red line indicates a negative correlation. Calprotectin was not part of the analysis in the circulation, it was included later in study II, see also Table 7. Illustration created in BioRender.

However, despite the presence of a systemic pro-inflammatory state in SLE, to our knowledge circulating cytokines as a measure of systemic inflammation are not included in any current DA indices [6,169,170].

1.5.1 Oral inflammatory markers in autoimmune diseases

The involvement of the mouth and oral mucosa in SLE is evident as the presence of oral ulcers is included in all three major sets of criteria for classification of SLE [15,19,20]. Dry mouth is another common oral manifestation, which is part of the classification criteria for SS [44].

In contrast to cytokines, autoantibodies are important tools in the classification, diagnosis, and monitoring of DA in autoimmune diseases. Autoantibodies such as RF, ACPA, and ANA, have been suggested to be involved in the pathogenesis of these conditions. However, it is worth mentioning that these antibodies can also be present in healthy individuals and that they commonly appear before disease onset [1,2]. Local production of SSA/SSB autoantibodies in GC-like structures within the salivary glands has been suggested [35,36]. This local production of autoantibodies has been subject to study to elucidate whether the subsequent development of autoimmunity occurs in the OC [53,54]. In SLE, SSA/SSB autoantibodies are common, usually stable over time and they appear early, even several years before disease onset [1,52]. An SLE phenotype characterized by these antibodies consistently appear in several studies [47-51].

The close relation between SLE, SS, and RA, overlapping and similarities open a door of opportunities to explore the eventual appearance of the typical SLE autoantibodies in the oral cavity besides the already explored SSA/SSB.

1.5.2 Biomarkers for periodontitis as predictors of autoimmunity

The idea of using accessible body fluids, such as serum, saliva, or gingivocrevicular fluid (GCF), as diagnostic tools for periodontitis (PD) has already been explored. The GCF for example, contains inflammation markers that can presumably predict the evolution of the disease [133]. However, many of these inflammatory markers are not specific for PD, and there is to date no serological biomarker capable of diagnosing PD or defining the subset of patients at increased risk of developing systemic diseases.

Previous studies have analyzed antibody responses to *Pg* as biomarkers. The existence of *Pg*-antigens recognized by serum IgG – with discriminating levels between PD patients as compared to non-PD – has been reported in recent years [171,172]. Hirai et al. identified 29 different components in *Pg* targeted by serum IgG, with antibodies towards *Pg* virulence factor arginine gingipain A (RgpA) proposed to be the most specific in identifying PD patients. Scientists at our research division have also shown significantly elevated levels of anti-RgpB IgG, not only in PD patients but also in RA patients compared to matched healthy controls, and in ACPA-positive *versus* ACPA-negative RA [149]. Arginine gingipains are *Pg*-specific proteases that cleave polypeptides after arginine, facilitating peptide-citrullination by the *Pg* PAD enzyme. The role of gingipains has been demonstrated in the pathogenesis of PD [134,173], important both for the survival of *Pg* and for its pathological effects [172,174].

1.6 Study rationale

PD has in recent years been described as linked to RA. Similarly, RA has many clinical features in common with SLE and SS and these conditions partly overlap regarding symptoms, antibodies, and risk genes. A high prevalence of SS in SLE and RA suggests that the involvement of the salivary glands and saliva may be important in the pathophysiology of these diseases. Consequently, the OC may have a potential role to increase our understanding of how autoimmunity occurs.

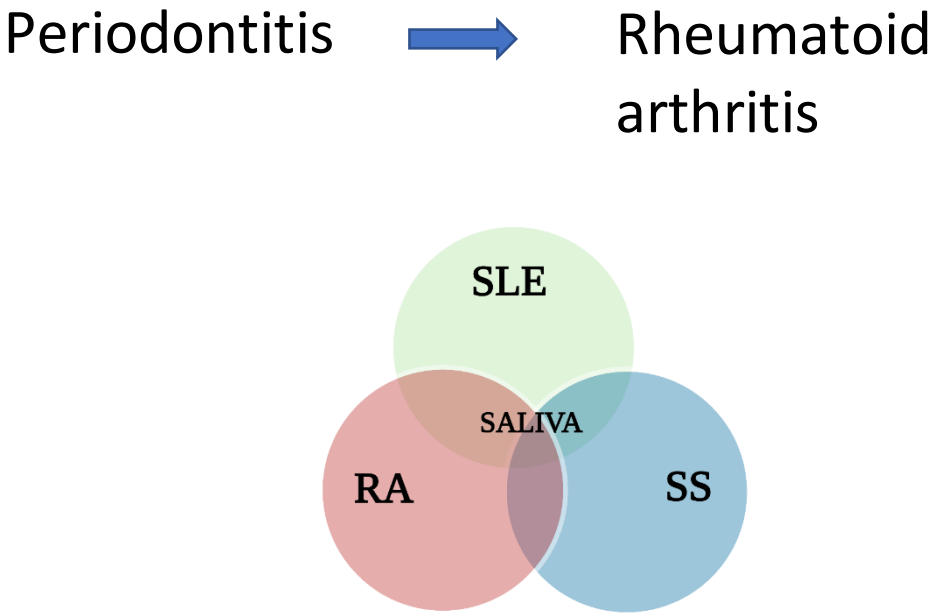


Figure 9. Sjögrens syndrome (SS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) relation and overlap. Venn diagram illustrating the Importance of saliva and the salivary glands in these diseases and the OC´s potential role to increase our understanding of how autoimmunity occurs.

The possibility to explore other body fluids that may be used to identify biomarkers is an opportunity to introduce non-invasive markers of inflammation in the DA indices. To our knowledge, there are no studies that have addressed this topic by the approach conducted in my thesis. Thus, the performed studies might contribute to the change of paradigms regarding the OC and its role in systemic conditions. Additionally, if PD severity correlates to the frequency of autoantibodies, it may also be a clue to understanding how autoimmunity occurs.

2 Research aims

2.1 General aims

The overall aim of this thesis was

i. to study the associations between oral factors, such as oral dryness, PD, and the presence of inflammatory biomarkers in saliva, and the autoimmune diseases SLE, SS, and RA,

ii. to study the relationship between well-defined PD and the presence of autoantibodies in three different groups of patients; the *PAROKRANK* multicentre case-control study, the *PerioGene North* case-control study, and the *SLE cohort*, and their respective matched controls, as well as

iii. to identify salivary biomarkers for autoimmunity linked to SLE and SS and their potential to evaluate disease activity as well as the pathophysiological mechanisms supporting these associations.

- Specific aims are stated for each outlined study below.

2.2 Specific aims

2.2.1 Study I

- To investigate the occurrence of sSS and its clinical and immunological differences and similarities in SLE patients and matched population controls.

- To study potential markers of systemic inflammation in patients with SLE-sSS *vs*. SLE-nonsSS.

2.2.2 Study II

- To explore the levels of innate immunity-related biomarkers in saliva, urine, and serum from SLE patients and population controls concerning measurements of DA.

- To evaluate the ability of these potential biomarkers to discriminate SLE patients from controls.

2.2.3 Study III

- To study whether the presence of autoantibodies is related to the occurrence or severity of periodontitis in three study populations: *PAROKRANK*, the *PerioGene North* case-control study, and SLE patients and controls.

- To investigate whether the antibodies towards *Pg* virulence factor arginine gingipain (Rgp) IgG could serve as a biomarker for periodontitis patients prone to develop autoimmunity.

2.2.4 Study IV

- To evaluate total Ig and specific autoantibody levels, in saliva from SLE patients and controls and to study the differences between the subgroups SLE-sSS and SLE-nonsSS, and how these measurements associate with each other.

3 Methods

3.1 Patients and controls

During the period February 2004 to December 2014, patients with SLE managed at the Department of Rheumatology, Karolinska University Hospital, and Danderyd´s Hospital (both Stockholm, Sweden) who fulfilled four or more items of the 1982 American College of Rheumatology revised classification criteria for SLE (n=504) were invited to participate in a large prospective cohort and be followed up longitudinally.

Population controls (n=319) were individually matched for sex, age, and geographic region to the first included SLE patients. The remaining SLE patients (n=185) did not have matched controls. Matching was performed by using of the national registration number, which includes the date of birth and is coded for sex. The only exclusion criteria for the controls were a diagnosis of SLE or SS. **Studies I - IV**.

During the period May 2010 to February 2014, patients hospitalized for a first MI at the coronary care unit in 17 Swedish hospitals, < 75 years old (n=805) were included in the *PAROKRANK* multicentre case-control study.

Exclusion criteria were a prior MI or prior heart valve replacement. Controls (n=805) were individually matched for sex, age, and geographic region. From the *PAROKRANK* study, individuals with non-missing data were selected for **study III**, comprising patients (n=779) and controls (n=719).

A third study population was included in **study III.** During the period 2009-2012 patients with periodontitis managed at the Specialist clinic for periodontology at Norrlands University Hospital, Umeå, Sweden, > 35 years old with severe periodontitis (n=41) were included in the *PerioGene North* case-control study.

Healthy controls (n=39) > 35 years old, were selected at the Public dental health clinic at Norrlands University Hospital, Umeå, Sweden. Exclusion criteria were, the use of antibiotics or periodontal treatment three months before inclusion, pregnancy or lactation, and any other disease or current anti-inflammatory medication, for both patients and controls.

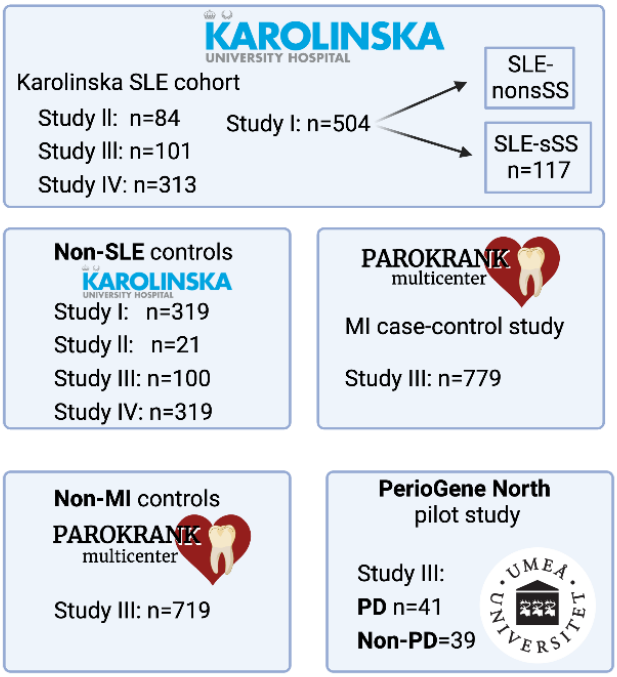


Table 4. Patients and Controls. Cohorts and studies, in which patients and controls were included.

3.2 Sample collection

3.2.1 Serum, urine, and saliva sampling

3.2.1.1 Serum

SLE patients and controls in **studies I - IV** were fasting for 12 hours before the collection of blood samples. Laboratory tests in serum were performed at the SWEDAC (www.swedac.se) accredited Clinical Chemistry and Immunology Laboratories at the Karolinska University Hospital.

In the *PAROKRANK* study **(study III)**, all participants were fasting and abstained from smoking 12 hours before blood sampling at the inclusion visit. The examinations were done 6-10 weeks after being hospitalized due to the MI with the expectation that the acute inflammation produced by the MI had subsided. Venous blood was collected for routine analysis and whole blood was stored at a biobank.

3.2.1.2 Urine

Urine was utilized for **study II.** At inclusion, all participants were fasting for 12 hours before collection of urine samples. The first-morning urine was collected and stored until analysis.

3.2.1.3 Saliva

In **studies I, II,** and **IV** at inclusion all participants were fasting for 12 hours before the collection of saliva samples.

The participants were asked to avoid tobacco consumption, brushing their teeth, and using lipstick one hour before saliva collection. WS was collected by passive drooling for 15 minutes, then placed on ice followed by centrifugation (335g x 10 min). The salivary flow (ml/min) was recorded.

Serum, urine, and saliva samples were stored at −70°C until analysis.

3.3 SLE disease activity

A variety of assessment tools is available for monitoring SLE DA. These tools are based on general and specific measures, monitoring methods of the organs and tissues, and evaluations of both the physicians and the patients. The DA indices are used both in clinical practice and for research purposes.

Three worldwide known and used indices for DA are the Systemic Lupus Activity Measure (SLAM) [175], the Systemic Lupus Erythematosus Disease Activity (SLEDAI) [176], and the British Isles Lupus Assessment Group (BILAG) [177], and we have based our measurements of DA in these in **study I** and **II**.

3.3.1 Systemic Lupus Activity Measure (SLAM) index

The SLAM is a standardized validated index, developed to measure global SLE disease activity. It is based on the events reported the last month and is scored on the severity of the indicators, with values reported as zero in the absence of a symptom or organ involvement.

Table 5. Systemic Lupus Activity Measure (SLAM) Index

CONSTITUTIONAL	***Mild-moderate***		***Severe***
1. Weight loss	1: < 10% body weight		3: > 10% body weight
2. Fatigue	1: Little or no limit on normal activity		2: Limits normal activity
3. Fever	1: 37.5 – 38.5º C or 99.5 – 101.3º F		3: > 38.5º C or > 101.3º
INTEGUMENT	***Mild***	***Moderate***	***Severe***
4. Oral/nasal ulcers, periungual, erythema, malar rash, photosensitive rash or nail fold	1:	1: With Trauma	2: Observed
5. Alopecia	1: With trauma	2: Observed	
6. Erythematous, macular or papular rash, discoid lupus, lupus profundus, or bullous	1: < 20% Total Body Surface Area (TBA)	2: 20 – 50% TBA.	3: > 50%TBA
7. Vasculitis (all)	1: < 20% TBA	2: 20 – 50% TBA.	3: > 50% TBA or necrosis
EYE	***Mild-moderate***		***Severe***
8. Cytoid bodies	1: Present		3: Visual acuity <20/200
9. Hemorrhages (Retinal or choroidal) or episcleritis	1: Present		3: Visual acuity <20/200
10. Papillitis or pseudotumor cerebri	1: Present		3: Visual acuity <20/200 or field cut
RETICULOENDOTHELIAL	***Mild***	***Moderate***	
11. Lymphadenopathy	1:	2: Diffuse or nodes > 1 cm x 1.5 cm	
12. Hepato- or Splenomegaly	1: Palpable only with inspiration	2: Palpable without inspiration	
PULMONARY	***Mild***	***Moderate***	***Severe***
13. Pleurisy / pleural effusion	1: Shortness of breath or pleuritic chest pain	2: Shortness of breath or pleuritic chest pain with exercise	3: Shortness of breath or pleuritic chest pain at rest
14. Pneumonitis	1: X-ray infiltrates only	2: Shortness of breath with exercise	3: Shortness of breath at rest
CARDIOVASCULAR			
15. Raynaud's phenomenon	1: Present		
16. Hypertension (diastolic pressure, mmHg)	1: 90 – 104	2: 105 – 114	3: > 115
17. Pericarditis/carditis	1: Pericarditis by ECG or effusion by echo	2: Positional chest pain or arrhythmia	3: Myocarditis with hemodynamic compromise and/or arrhythmia
GASTROINTESTINAL			
18. Abdominal pain (serositis, pancreatitis, or ischemic bowel, etc)	1: Complaint	2: Limiting pain	3: Peritoneal signs /ascites
NEUROMOTOR			
19. Stroke syndrome, includes mono neuritis multiplex (MM), reversible neurologic deficit (RND), cerebrovascular accident	1: TIA	2: RND, MM, cranial neuropathy, or chorea	3: CVA, myelopathy, or RVO

(CVA), or retinal vascular occlusion (RVO)			
20. Seizure		2: 1 or more per month	3: Status epilepticus
21. Cortical dysfunction	1: Mild depression, personality disorder, or cognitive deficit	2: Change in sensorium, severe depression, or limiting cognitive impairment	3: Psychosis, dementia, or coma
22. Headache (including migraine equivalents and aseptic meningitis)	1: Symptoms only	2: Interferes with normal activities / aseptic meningitis	
23. Myalgia / Myositis	1: Symptoms only	2: Limits some activity	3: Incapacitating
JOINTS			
24. Joint pain	1: Arthralgia only	2: Objective synovitis	3: Limits function
LABORATORY			
25. Hematocrit (mg/dL)	1: 30 – 35	2: 25 – 29	3: < 25
26. White blood cell count (per mm^3)	1: 2000 – 3500	2: 1000 – 1999	3: < 1000
27. Lymphocyte count (per mm^3)	1: 1000 – 1499	2: 500 – 999	3: < 500
28. Platelet count (x 1000 per mm^3)	1: 100 – 149	2: 50 – 99	3: < 50
29. Westergren ESR (mm/hr)	1: 25 – 50	2: 51 – 75	3: > 75
30 Serum creatinine (mg/dL) or creatinine clearance (% normal)	1: 1.4 – 2.0 or 60 – 79%	2: 2.1 – 4.0 or 30 – 59%	3: > 4.0 or < 30%
31. Urine sediment (per high power field)	1: 6 – 10 RBC or 6 – 10 WBC; OR 0-3 granular or 0-3 non-RBC casts; OR trace – 1+ protein (3 granular or >3 non-RBC casts; OR 2 – 3+ protein (>500 mg – 3.5 g/L24 hr urine protein)		3: > 25 RBC or > 25 WBC; OR any RBC casts; OR 4+ protein > 3.5 g/L24 hr urine protein)

The SLAM Index consists of 24 clinical manifestations and 7 laboratory parameters. No immunological parameters are included [178].

3.3.2 Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)

The SLEDAI was introduced and validated in 1985 and is perhaps the most used DA index. It is based on the events reported in the last ten days and is scored about severity of the indicators.

The SLEDAI 2000 (SLEDAI-2K), is a revised version of SLEDAI, introduced in 2002 [179]. In contrast to the SLEDAI, the SLEDAI-2K, evaluates persistent activity in specific items. Whereas in the original SLEDAI only new onset of symptoms are evaluated.

Table 6. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)

Weight	Descriptor	Definition
8	Seizure	Recent onset. Exclude metabolic, infectious, or drug-related causes.
8	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Includes hallucinations; incoherence; marked loose associations; impoverished thought content; marked illogical thinking; bizarre, disorganized, or catatonic behavior. Exclude the presence of uremia and offending drugs.
8	Organic brain syndrome	Altered mental function with impaired orientation or impaired memory or syndrome other intellectual function, with rapid onset and fluctuating clinical features. Includes a clouding of consciousness with a reduced capacity to focus and an inability to sustain attention on the environment, and at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, and increased or decreased psychomotor activity. Exclude metabolic, infectious, and drug-related causes.
8	Visual	Retinal changes from systemic lupus erythematosus: cystoid bodies, retinal hemorrhages, serous exudates or hemorrhages in the choroid, optic neuritis (not due to hypertension, drugs, or infection).
8	Cranial nerve	New onset of a sensory or motor neuropathy involving a cranial nerve.
8	Lupus headache	Severe, persistent headache; may be migrainoid; unresponsive to narcotics.
8	CVA	New syndrome. Exclude arteriosclerosis.
8	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages. Vasculitis confirmed by biopsy or angiogram.
4	Arthritis	More than 2 joints with pain and signs of inflammation.
4	Myositis	Proximal muscle aching or weakness associated with elevated creatine phosphokinase/aldolase levels, electromyographic changes, or a biopsy showing myositis.
4	Urinary casts	Heme, granular, or erythrocyte.
4	Hematuria	More than 5 erythrocytes per high power field. Exclude other causes (stone, infection).
4	Proteinuria	More than 0.5 grams of urinary protein excreted per 24h. New onset or recent increase of > 0.5 g/24h.
4	Pyuria	More than 5 leukocytes per high-power field. Exclude infection.
2	Malar rash	New onset or recurrence of an inflammatory type of rash.
2	Alopecia	New or recurrent. A patch of abnormal, diffuse hair loss.
2	Mucous membranes	New onset or recurrence of oral or nasal ulcerations.
2	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	Pericarditis	Pericardial pain with at least one rub or effusion. Confirmation by electro- or echocardiography.
2	Low complement	A decrease in CH50, C3, or C4 level (to less than the lower limit of the laboratory-determined normal range).
2	Increased DNA binding	More than 25% binding by Farr assay (to >the upper limit of the laboratory-determined normal range, e.g., 25%).
2	Fever	More than 38 ºC after the exclusion of infection.
2	Thrombocytopenia	Fewer than 100,000 platelets
2	Leukopenia	Leukocyte count of < 3000/mm3 (not due to drugs)

The SLEDAI includes immunology features and is based on the presence of 24 items in nine organ systems [176].

3.4 Quartiles of disease activity

In **study II,** we were interested in patients with high and low disease activity (DA). Patients with missing data from either the SLAM or the SLEDAI-2K indices were excluded. One group with low DA (SLAM <7 and SLEDAI-2K <4; n=50) and one group with high DA (SLAM >7 and SLEDAI-2K >4; n=63) were identified. Accordingly, a third group (n=181), was discordant for DA by SLAM and SLEDAI-2K, and this group was excluded.

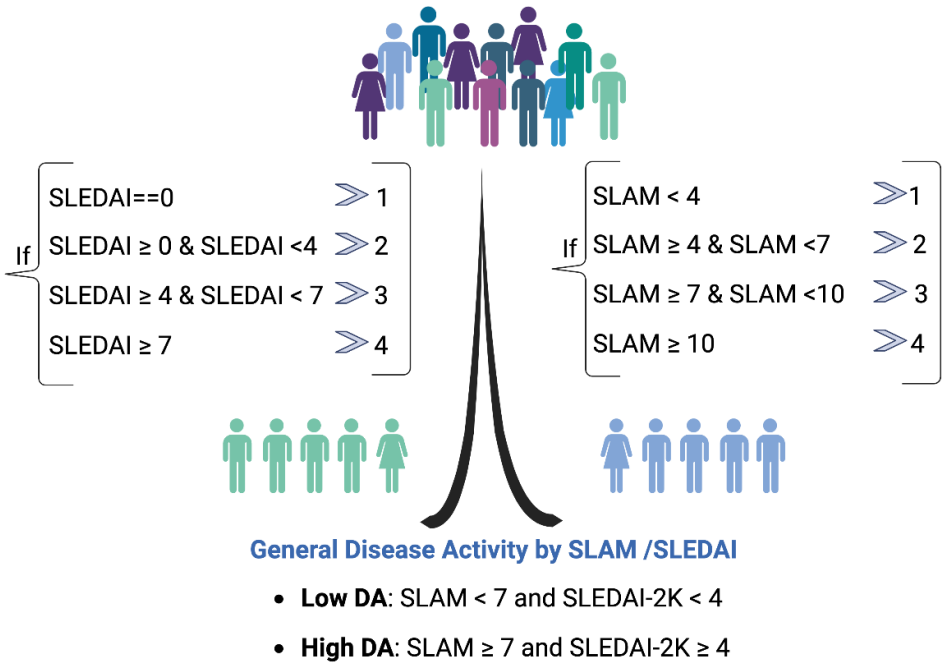


Figure 10. Quartiles of disease activity (DA). We selected patients from the high- (**n=42**) and from the low- (**n=42**) DA groups, and controls (**n=21**) with a relatively similar and normally distributed age for final inclusion to avoid age over-representation in any of the groups. We also included a similar number of gender representations in each group. Created in Biorender.

3.4.1 Renal disease activity

In **study II,** renal DA was defined according to the BILAG index. The BILAG is another SLE index, broadly used, particularly in clinical trials. The BILAG assesses eight organs and weighs their severity. Each organ also includes domains to assess the whole organ system, with help of laboratory and clinical tests combined into a single score [177].

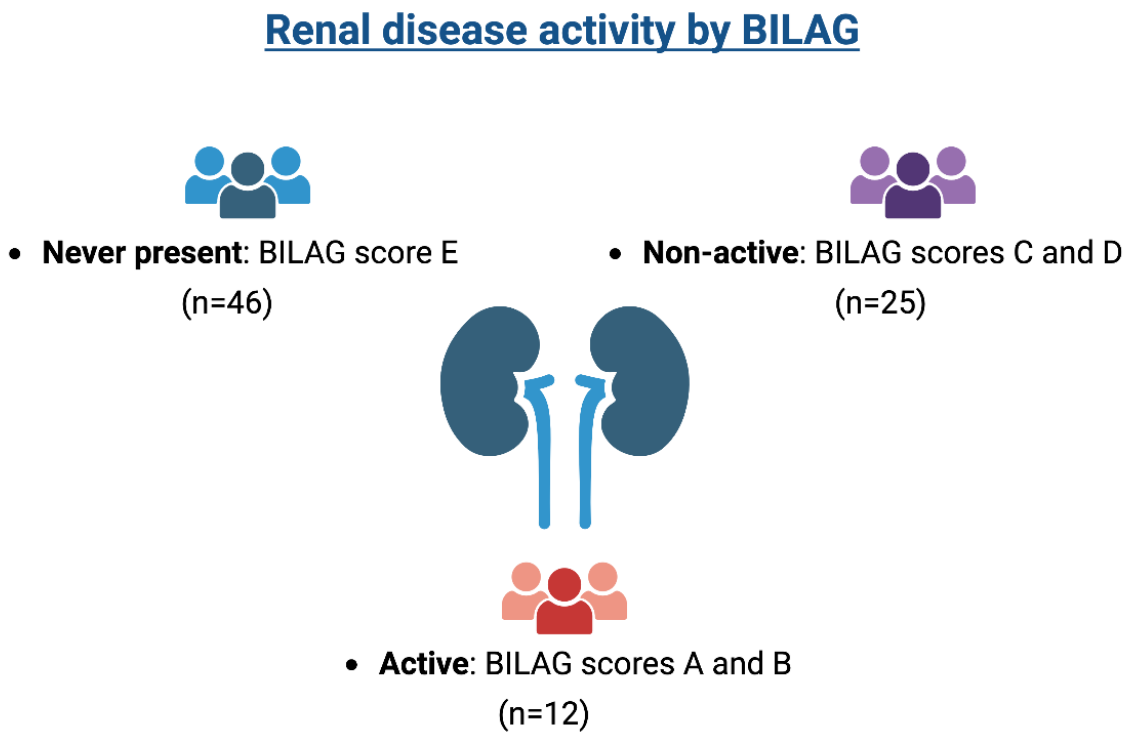


Figure 11. Renal disease activity. The scoring scale in the BILAG index is based on letters, and ranges from A to E; A indicating maximal disease activity and E indicating no prior involvement of the organ domain. Created with Biorender.

3.5 Periodontal examinations

For **study III,** periodontal status was not available for the SLE cohort, though we have started the recruitment of potential periodontal patients through a questionnaire. These parameters are part of the follow-up data planned to be included in **study IV**.

In **study III**, in the *PAROKRANK* study, periodontitis was defined based on mean bone loss of all teeth measured in panoramic radiographs; calculated from the marginal bone to the tooth apex (total bone height) and from the cement-enamel junction to the tooth apex (total root length) [73]. Three groups were classified: no periodontitis (>80% remaining bone), mild to moderate periodontitis (66-79% remaining bone), and severe periodontitis (<66% remaining bone). BOP was measured at four sites per tooth, and the BOP index was calculated based on the total number of measured sites. BOP was categorized in grades 0, 1, or 2, with a BOP index of 0-9.9%, 10.0-29.9%, and ≥30%, respectively.

In **study III**, in the *PerioGene North*, severe periodontitis was defined when >15 teeth remained, periodontal damage in $\geq$ 50% of teeth with >1/3 bone loss of the root length, and BOP at >20%. Whereas the periodontally healthy controls were considered as such when ≥24 teeth remained, absence of CAL and PPD <4 mm at all sites. BOP was measured at six different sites per tooth, in the *PerioGene North* case-control study.

3.6 Immunoassays

3.6.1 Enzyme-linked immunosorbent assay *(ELISA)*

ELISA is a plate and antibody-based assay technique. It detects and quantifies antigens in biological fluids such as serum, plasma, saliva, urine, and cell culture supernatants e.g., viruses, antibodies, cytokines, lipids, and carbohydrates. Several types of *ELISA* are available: direct-, indirect-, "sandwich"-, or competitive-*ELISA* **(Figure 12)**.

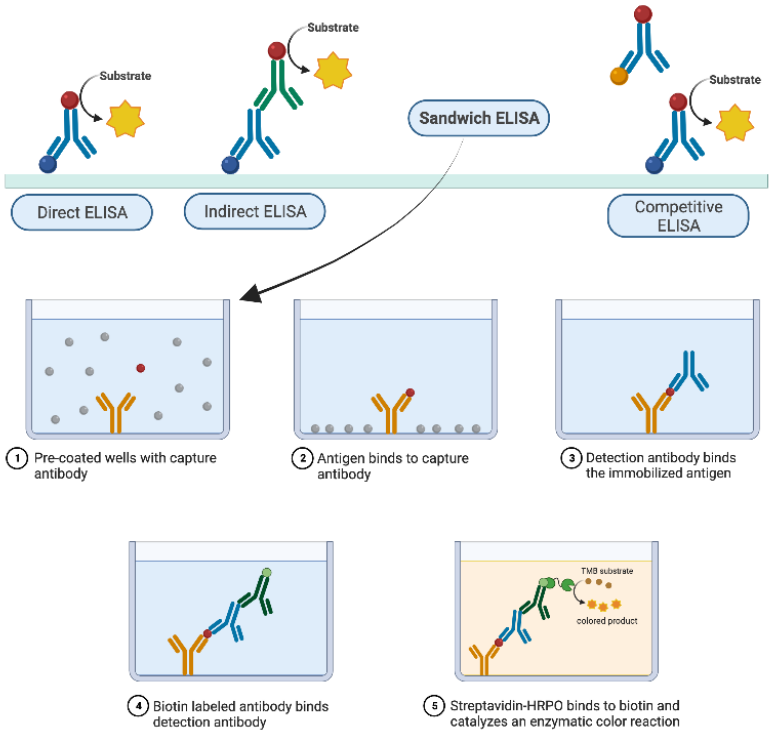


Figure 12. *ELISA*. Illustration of different *ELISA* techniques: **(i)** direct *ELISA*; when a primary antibody conjugate only one detection antibody, it detects soluble antigens and plates are coated with sample or antigen and detected with antibody-enzyme conjugate, as **(ii)** "sandwich" *ELISA;* when two specific antibodies "sandwiches" the antigen, a capture antibody for the investigated protein is coated before adding the sample and an enzyme-conjugated-detection antibody binds to an additional epitope on the target protein. Since two antibodies are required to bind to the protein of interest they are deemed as highly specific [180]. Created with Biorender.

In **study II,** for CSF-1, IL-34, and calprotectin levels, we used commercial *ELISA* kits *(R&D Systems, Minneapolis, Minnesota, USA)* to measure protein concentrations in saliva, serum, and urine from SLE patients and controls. In **study IV** for total IgA, total IgG, and anti-dsDNA IgG levels, we used commercial *ELISA* kits *(Abcam, Cambridge, UK & Orgentec diagnostika GmbH, Mainz, Germany)* to measure protein concentrations in saliva, from SLE patients and controls. In **study III,** serum samples were analyzed by an in-house *ELISA* for the presence of anti-Rgp IgG, as previously described [181].

3.6.1.1 Bead-based multiplex immunoassays

Similar to a "sandwich" *ELISA*, the beads are added to the wells, followed by the samples. The utilization of a secondary antibody "sandwiches" the antigen. The magnetic beads are dyed and each of them discriminates between the different analytes. In **study II;** TNF-α, MCP-1, IP-10, macrophage inflammatory protein (MIP)-1α and MIP-1β, and in **study IV;** anti-SSA/Ro52, anti-SSA/Ro60, anti-SSB/La, anti-smith (Sm), anti-Ribonucleoprotein (RNP), the Sm/RNP complex, anti-Ribosomal P, antigen β2-glycoprotein1 (aβ_2GP1), and anti-C1q, were analyzed using a commercial bead-based multiplex immunoassay.

3.6.2 Testing, optimization, and validation

3.6.2.1 Spike-and-recovery assessment

Spike-recovery-and-linearity is a method for validating and assessing the accuracy of *ELISA*. It is used to determine whether analyte detection is affected by differences in the standard curve diluent and biological sample matrix, in this case, saliva.

In **study IV** for validation of the dsDNA IgG kit for saliva, samples were diluted to evaluate at which dilution the saliva matrix reach linearity at the range of the standard curve without interference.

3.6.2.2 Total protein

Determination of the total amount of protein was assessed in **study II** in all saliva and urine samples but not in serum with Pierce BCA Protein Assay Kit (*Thermo Fisher Scientific, Waltham, MA, USA).*

3.6.3 Autoantibodies in serum

In **studies I, II, III,** and **IV** the SLE and *PAROKRANK* investigated subjects were screened for ANAs; antibodies to specific nuclear antigens (dsDNA, SSA-Ro52, SSA-Ro60, SSB/La, Sm, RNP), and phospholipid- related antigens (cardiolipin (CL) IgG, IgM, IgA, and aβ_2GP1 IgG, IgM, IgA) as well as ACPA (detected as anti-cyclic citrullinated peptide 2 (CCP2) IgG).

The cut-off for anti-CL and aβ_2GP1 fulfills the 99th percentile of the general population in Stockholm, as described by Miyakis et al. [76]. Lupus Anticoagulant was determined using a modified Dilute Russel Viper Venom method (Biopool, Umea, Sweden) using Bioclot lupus anticoagulant.

RF (non-class specific) was measured by nephelometry for the screening of the *PAROKRANK* group in **study III**. In the SLE cohort, IgA, IgG, and IgM RF were analyzed by fluorescence enzyme immunoassay using the EliA system. Cutoffs for RF isotypes were determined as >95% specificity compared with 100 blood donors for IgA and IgM RF and for 285 population controls for IgG RF.

3.6.4 Cytokines in serum

In **study I** *Mesoscale Discovery (MSD)* multiplex analysis of cytokines was performed on EDTA-plasma samples were analyzed on the MSD V-PLEX™ Human Cytokine 30-plex kit *(K15054D; Mesoscale Discovery, Gaithersburg, MD).*

3.7 Statistical analyses

Patient and control characteristics were presented as mean ± standard deviation (SD), median (interquartile range, IQR), or percentages, depending on data type and distribution.

Groups were compared with the Student´s t-test, Kruskal-Wallis, Wilcoxon rank sum test, Chi-square, or Fisher´s exact tests as appropriate. When multiple groups were compared, a Dunn-Bonferroni post hoc test was performed. We used non-parametric tests when the log transformation of continuous variables did not give an approximately normal distribution. Adjustment for age was performed by multiple logistic regression.

Calculations were performed using Statistical Package for Social Sciences (SPSS), version 24 or 26, JMP software, version 13.0, Prism, version 8 and R, version 4.1.1. A two-sided p-value < 0.05 was considered statistically significant.

In **studies II** and **IV,** correlations between the biomarkers and the clinical variables as well as the correlations between the body fluids in **study II** were determined by the Spearman rank correlation coefficient, as well as the correlations between the body fluids in **study II**.

In **study III,** continuous variables were presented as median, with the 10th-90th percentile range (box plot graphs) or with minimal and maximal values (tables), and categorical variables as frequencies.

In **studies II** and **III,** to evaluate the accuracy of the biomarkers to discriminate patients from controls, the area under the receiver-operating characteristic curves (AUROC) and 95% confidence intervals (CI) were calculated for each marker. In **study III,** Youden´s J statistic was used to determine the highest sensitivity and specificity. In **study III,** matched analyses (where we matched individuals based on age, sex, and smoking) were performed using coarsened exact matching with the Match-It package in R.

3.8 Ethical considerations

The local Ethics Committee at Karolinska Institutet in Stockholm County Stockholm approved Dnr 2008/152-31/2 *PAROKRANK*, Dnr 03-556, Dnr 2017/1570–32 SLE cohort, Dnr 2020-04566 *PerioGene North* for **studies I, II, III,** and **IV**. All study subjects gave written informed consent to participate in the study.

Data and personal information of the included patients are managed during the performance of these studies. Only participants who had given written informed consent to participate were included in the outlined studies. Personal data was always coded during data analyses. In the publication of the results, all participants were presented as groups, and not as individuals.

The present research project has implications for two statements of the Declaration of Helsinki: the benefit of human society by understanding the causes of disease, improving diagnostic interventions, and protecting the privacy and confidentiality of each of the participants (6th and 24th principles, respectively).

This project has as main focus the study of oral factors related to autoimmunity, particularly to SLE and also RA (**study III**). SLE is a systemic autoimmune disease, which because of its heterogeneity and the involvement of several organs, has been considered a prototype for autoimmune diseases. Therefore, it is an opportunity to develop new knowledge that can be used not only in this disease but also in other related diseases. We have chosen to study the oral cavity in SLE patients and to investigate potential biomarkers that might aid to monitor DA in SLE. By the principle of justice, we will gain knowledge, which has the potential to benefit both patients with SLE and patients with other diseases, which the oral cavity is involved.

It is noteworthy to mention that this SLE cohort was initially recruited many years ago, and there is stored blood, saliva, and urine in the Biobank at Rheumatology Karolinska. The patients gave their informed consent to donate these body fluids, ensuring that the principle of autonomy was respected.

In conclusion, this thesis is based and strongly tied to the highest standards of ethics on research which are fundamental when conducting scientific protocols at the Karolinska Institute.

3.8.1 Gender perspective

Autoimmunity in general is more common among women than among men. SLE and SS are diseases with a strong female predilection (90%). To get statistically significant results, we always need a sufficient number of patients and in this case, the low frequency of men (10%) indicates the necessity of larger cohorts to analyze gender-specific differences. Our SLE cohort is considered large even from an international perspective. Therefore, we count on the possibility to perform gender stratified analyses in SLE.

In the *PAROKRANK* study, 81% of the participants were men. The incidence of a first myocardial infarction is more common in men than in women if we consider our inclusion criteria for patients <75 years old. The *PAROKRANK* study was also large, and we were therefore powered to perform gender-stratified analysis.

4 Results and discussion

In **studies I – IV**, we evaluated cytokines and autoantibodies in SLE patients to understand their involvement in the disease and the possible contribution of the oral cavity to pathophysiological mechanisms of autoimmunity. In **study III**, we explored the presence of autoantibodies, which are typically observed in SLE and RA, in patients with PD in order to explore the link between the periodontal opportunistic pathogen *Pg,* and autoimmunity. In **studies I – IV**, we assessed several proteins for their potential as biomarkers for DA in SLE, and in the case of **study III** the identification of a PD subset prone to develop into autoimmune disease **(Table 7).**

Table 7. Investigated biomarkers in the different studied diseases included in this thesis

Studies	Study I - IV				Study II	Study III	
Disease	**SLE**		**SLE-sSS**		**SLE**	**Periodontitis**	
Fluid	**Serum**	**Saliva**	**Serum**	**Saliva**	**Urine**	**Serum**	**Saliva [§]N/A**
Antibodies							
Total IgG	*	*	*	*			
Total IgA	*	*	*	*			
Total IgM	*		*				
Anti-Rgp IgG	*					*	
Autoantibodies							
anti-dsDNA	*	*	*	*			
anti-SSA-Ro52	*	*	*	*			
anti-SSA-Ro60	*	*	*	*			
anti-SSB-La	*	*	*	*			
RF IgG	*		*				
RF IgA	*		*				
RF IgM	*		*				
anti-RNP	*	*	*	*			
anti-Sm/RNP	*	*	*	*			
anti-Ribosomal	*	*	*	*			
anti-β2-glycoprotein1	*	*	*	*			
anti-C1q	*	*	*	*			
Cytokines							
TNF-α	*	*	*	*	*		
IL-6	*		*				
MCP-4	*		*				
MIP-1	*	*	*	*	*		
MIP-1β	*	*	*	*	*		
IL-12/23p40	*		*				
IP-10	*	*	*	*	*		
Calprotectin	*	*	*	*	*		
CSF-1	*	*	*	*	*		
MCP-1	*	*	*	*	*		
IL-34	*	*	*	*	*		

Table indicating the investigated biomarkers in the different patient groups in serum, saliva, and urine presented and discussed in this section.

[§] = not available

4.1 Oral manifestations of autoimmune diseases

4.1.1 Sjögrens syndrome secondary to SLE (study I)

In **study I** we demonstrated that, in contrast to lower frequencies reported earlier [55-59], secondary SS to SLE occurs in roughly ¼ of SLE patients when strictly applying the AECC [44]. In line with what has already been reported [55-57,182,183], we demonstrated that the SLE-sSS subset was older at inclusion (54.6±13.6 vs. 43.4±14.7 years; p= <0.0001) and at SLE onset (40.4±15.6 vs. 31.9±14.9 years; p= <0.0001) as compared with the SLE-nonsSS group. These findings may be the result of a gradual degradation of the salivary glands, which could be subclinical and ongoing for many years in the SLE-sSS group before receiving a diagnosis [60]. Although, our group has earlier demonstrated a strong association between SLE and the *HLA-DRB1*03* alleles, which indicates that a genetic predisposition also makes an important contribution to sSS in SLE [46]. Nonetheless, in **study I** subjective symptoms differed between SLE-nonsSS and controls in all age spans, while differences were small for WUSF when age >50 years, strengthening the observation that sSS is an age-related complication among patients with SLE [55,60].

Moreover, in all the investigated groups in **study I** (SLE-sSS, SLE-nonsSS, and controls), subjective symptoms of ocular and/or oral sicca symptoms were less frequent than the objective measurements. In **study I**, organ damage was more severe in the SLE-sSS group, presumably due to the inflammatory state demonstrated by the elevated pro-inflammatory cytokines (TNF-α; p=0.008, IL-6; p=0.009, MCP-4; p=0.019, MIP-1β; p=0.020, IL12/IL-23p40; p=0.031, and IP-10; p=0.036) upregulated in SLE-sSS vs SLE-nonsSS. The silent degradation of the salivary glands has shown us that the damage is progressive. Therefore, it is important to investigate why the patients do not experience these symptoms to avoid clinical consequences such as oral candida, disturbance in the healing of the soft tissues as well as tooth decay because of the reduced salivary flow.

Taken together, considering the low subjective report of the sicca symptoms, it would be of clinical relevance to identify biomarkers as an aid to performing more objective measurements, and prevent further unnecessary oral comorbidities in SS.

4.1.2 Oral ulcers in SLE (study I, II, and IV)

The involvement of the mouth and the oral mucosa in SLE is evident, and the presence of oral ulcers is one of the original 11 criteria to classify SLE, and oral ulcers are included in all three major sets of criteria for the classification of SLE [15,19,20]. Accordingly, in **study I** we tested whether the presence of oral ulcers positively correlated with positivity to anti-SSA/Ro52, anti-SSA/Ro60, and anti-SSB/La. Conversely, with previous reports [184], which showed strong associations, we didn't find any statistically significant result (35.7%; p=0.46, 33%; p=0.87 and 35.4; p=0.58, respectively).

In **study II,** only twenty-two patients with SLE presented with oral ulcers at the time of inclusion (n=13 in the high DA and n=9 in the low DA group) but CSF-1, TNF- α, IP-10, and MCP-1 in saliva did not differ between patients with SLE with- as compared with without- oral ulcers (data not shown). Overall, our data doesn't show any associations between oral ulcers and the presence of autoantibodies in **study I**, while in **study II,** lack of power leaves our comparisons inconclusive.

In **study IV,** when analyzing a larger number of patients, differences between the levels of total salivary IgA and IgG in individuals with a history of oral ulcers (n=111 *vs.* 511) were analyzed. Total salivary IgA levels were higher in the individuals with a history of oral ulcers, and salivary IgG was also higher in this subset (p=<0.05). An explanation could be that IgA is the most abundant Ig in the oral cavity, ascribed to its mucosal involvement [35,82,185], and that IgA antibodies can induce all IgG subclasses by Fc-receptors [35].

4.1.3 Periodontitis–an oral manifestation of autoimmunity (study III)

PD has been shown to be associated with autoimmune diseases such as RA and SLE [86,88,186], but the underlying mechanisms are still being explored and whether a causal relationship exists remains to be shown. In **study III**, we investigated RA and SLE autoantibodies in relation to periodontal status in the *PAROKRANK* cohort [73]. This cohort was comprised of individuals with a first myocardial infarction (n=779) and matched controls (n=719), where 557 had periodontitis.

Initially, we performed an explorative analysis of the dataset. Signature antibodies for RA, ACPA, and RF [187,188] (levels and positivity), were tested in PD patients *versus* non-PD controls. Of note, as this was not an RA cohort, only 3.5% were positive for ACPA and 4.7% for RF. Still, our analysis showed a trend towards a higher frequency of ACPA positivity among the PD patients (**p=0.06**), which was not observed in RF positive patients. Also, ACPA levels, but not RF levels, were higher in PD *versus* non-PD, (p=<0.01), **(Figure 13).**

In addition, ACPA and RF levels were tested concerning pocket depth ≥6mm as compared with those <6mm pocket depth, again only ACPA levels showed a trend for higher levels in the ≥6mm group *vs.* the <6mm group (**P=0.06**). Hence, our data suggest a closer relationship between ACPA and PD than between RF and PD.

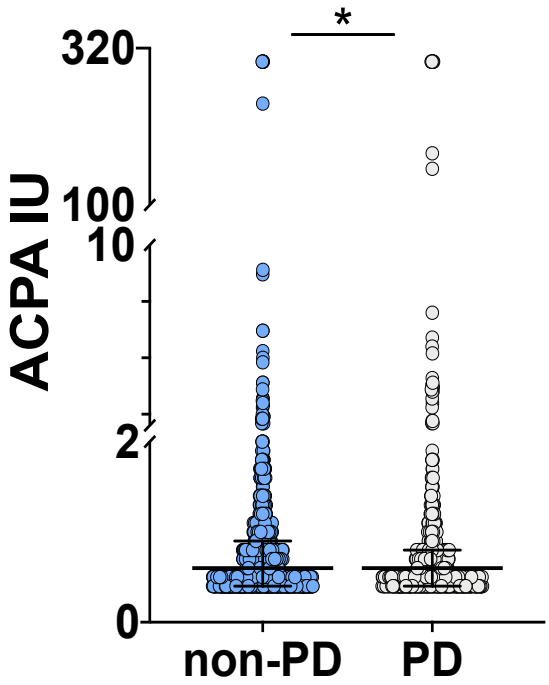


Figure 13. ACPA levels in non-periodontitis (non-PD; n=940), and Periodontitis (PD; n=552) groups of *PAROKRANK* patients. Median-IQR.. Mann-Whitney test. P = <0.01.

However, these data were mainly non-significant, so we proceeded to analyze ACPA, RF, and SLE-associated autoantibodies concerning anti-*Pg*-Rgp antibodies in PAROKRANK.

In **study III**, and in line with earlier studies [149,171,189], we confirmed significantly higher levels of Rgp IgG in PD *versus* non-PD, and we showed that Rgp IgG autoantibodies were primarily associated with PD severity. Previously, research within our division has shown that anti-RgpB IgG levels were significantly elevated not only in PD but also in ACPA-positive RA patients compared to ACPA-negative RA patients [149]. However, these data were not confirmed elsewhere, though associations between ACPA / RF and antibodies against another periodontal pathogen, *Prevotella intermedia*, were demonstrated [153]. In **study III**, when analyzing anti-Rgp IgG levels with RA autoantibodies, including both RF and ACPA, in the *PAROKRANK* study, we found significantly higher Rgp IgG levels in RA autoantibody-positive compared to -negative individuals (P=<0.05). Furthermore, when the comparison was made with only ACPA or only RF, ACPA was significantly elevated (P=<0.05), but not RF. Hence, our data support a role for *Pg* in ACPA+ RA and, a current hypothesis that *Pg* may trigger and drive the ACPA response by mechanisms of molecular mimicry [190], and that the underlying mechanisms behind the PD-RA link involves recurrent bacterial metabolites enter the circulation, constantly activating ACPA-positive B cells [37].

In **study III,** we also analyzed some SLE-associated autoantibodies in relation to Rgp IgG, and we found that anti-Rgp IgG levels were significantly increased in the group positive for anti-dsDNA antibodies (P=<0.05). Consequently, we confirmed this finding in a separate cohort of SLE patients (n=62 dsDNA IgG positive; n=39 dsDNA IgG negative), p=<0.05. Notably, there was no difference in levels of anti-Rgp IgG in SLE patients compared to controls, and there was also no difference in Rgp IgG levels when subgrouping SLE patients based on other SLE-associated autoantibodies. Our data are in line with earlier reports demonstrating that anti-dsDNA positive SLE patients were enriched in high antibody levels to *Pg* and other PD-associated bacteria [117].

Taken together, our findings suggest a potential role for Rgp IgG as a biomarker for PD patients at increased risk of developing specific autoimmunity linked to RA and SLE. Moreover, based on our data we speculate that the molecular mechanisms linking *Pg* to ACPA/dsDNA IgG may relate to the nature of the two autoantigens targeted specifically by ACPA and dsDNA antibodies. Because both citrullinated proteins and dsDNA are released during neutrophil extracellular trap formation (NETosis), and PD is characterized by excessive NET formation – efficiently triggered by *Pg* Rgp – and impaired NET clearance, leading to exposure of dsDNA and citrullinated proteins in the context of bacterial danger molecules, which may facilitate the loss of tolerance and production of ACPA or dsDNA antibodies in genetically susceptible individuals [191,192].

*4.1.3.1 Cardiovascular diseases–A cause or a consequence (**study III**)*

In **study III**, we also report significantly increased levels of Rgp IgG in patients who had a first MI compared to matched controls, in line with previous data from the group, demonstrating a close relationship between MI and PD in the *PAROKRANK* cohort [73]. However, within the PD group, Rgp IgG levels could not discriminate those with MI from those without.

In **study III** we screened the *PAROKRANK* study for ANA subspecificities but also for aPL. Also, in our group, we have previously reported significantly higher levels of aPL of the IgG isotype in MI *vs.* controls [29] in the *PAROKRANK* study. Additional analysis with only PD-positive MI patients (n=307) *vs*. non-MI (n=250) were performed; the levels of aPL of the IgG isotype remained significant strengthening the association of PD with both MI and the presence of aPL (p=<0.01) **(Figure 14).**

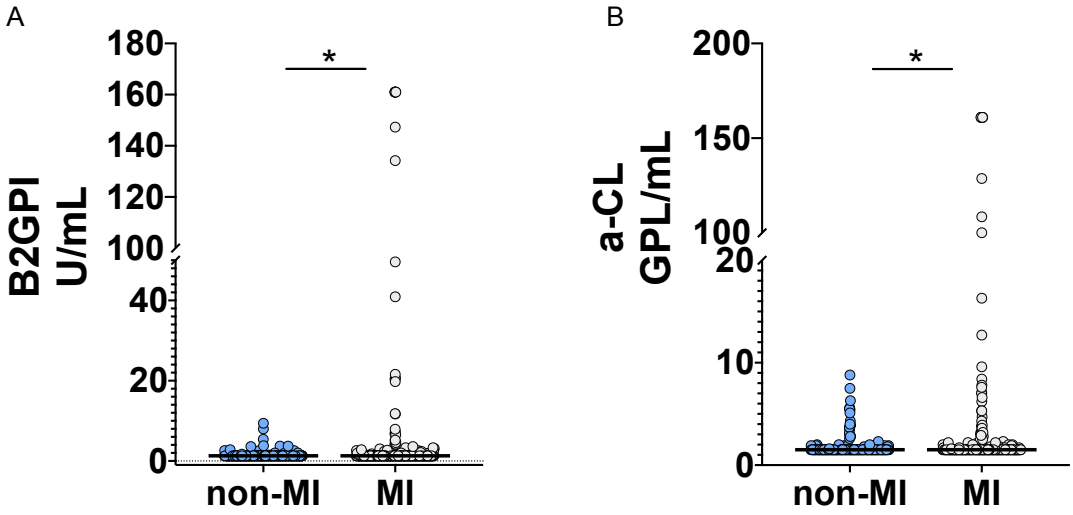


Figure 14. Levels of anti-β2-GPI (A) and anti-CL (B) of the IgG isotype in periodontitis positive- healthy controls (non-MI; n=250), and -myocardial infarction (MI; n=307) groups of *PAROKRANK* patients. Median-IQR. Mann-Whitney test. P = <0.01.

4.2 Biomarkers for oral involvement in autoimmune diseases

4.2.1 Serum biomarkers

The potential of serum anti-Rgp as biomarker to identify a subset of PD patients, with an enhanced risk to develop autoimmunity linked to RA and SLE, was supported by our data. Other markers for oral involvement in autoimmunity will be presented next. Both, well-established biomarkers for other diseases with oral involvement, as is the case for SS, as well as novel biomarkers, which were proposed as proxies for inflammation or DA, were tested in serum and alternative non-invasive fluids.

4.2.2 Serum cytokines and autoantibodies

*4.2.2.1 Cytokines (**study I** and **II**)*

In **study I,** despite less internal organ involvement, we found a pro-inflammatory state in the SLE-sSS group. Of 20 detected cytokines, six were higher (TNF- α, IL-6, MCP-4, MIP-1β, IL12/IL-23p40, and IP-10) in the SLE-sSS (n=117) than in SLE-nonsSS (n=387) group (all p=<0.05). Other authors have reported that some of these cytokines are present in SLE [193,194], but only a few studies have explored the role of circulating cytokines in pSS [163,195]. Furthermore, they reported numerous associations with clinical and laboratory parameters. Conversely, several studies have observed cytokine profiles in pSS histopathologically [162,196,197].

Our discoveries, relate to the findings in circulation by Szodoray et al. [163] where, apart from MCP-4 and IP-10, they also found IL-6, IL-12p40, TNF- α, and MIP-1β upregulated in SS vs. ctrls. Based on these results we investigated the levels of some cytokines in SLE patients in **study II**. Overall, our findings confirm the presence of pro-inflammatory cytokines in SLE and demonstrate their potential as biomarkers for DA.

*4.2.2.2 Autoantibodies (**study I**)*

In **study I,** we demonstrated that despite not being included in the 2002 AECC [44]; anti-SSA-Ro52, anti-SSA-Ro60, and anti-SSB/La autoantibodies are more frequent in the SLE-sSS subset [49,52] (47.9% vs. 21.8 %; p= <0.0001, 59% vs. 35.9%; p= <0.0001 and 37.6% vs. 18%; p= <0.0001, respectively).

We also compared the relationship of anti-SSA/SSB autoantibodies with oral sicca symptoms in **study I.** In the SSA-Ro52, SSA-Ro60 and SSB/La positive groups; 49.2%, 52.7%, and 50.9 % respectively, presented with subjective oral sicca symptoms. Figures were similar for the triple SSA-/SSB-positive patients (53.9 %).

Altogether, our findings support the existence of an anti-SSA/SSB cluster among patients with SLE and this cluster is enriched in sicca symptoms [47,48,198]. Furthermore, it is notable that subjective sicca symptoms were less often reported than verified objective sicca measurements. Haldorsen et al. [194] concluded, that SSA seropositivity may foresee degradation in salivary gland function, but if anti-SSA/SSB autoantibodies are present before the onset of sicca symptoms deserves further investigation.

4.2.3 Saliva biomarkers

*4.2.3.1 Total salivary IgA and IgG (**study I** and **IV**)*

Excess production of Ig in SS leads to hypergammaglobulinemia in many patients with SLE-sSS, a well-known characteristic in this subset [198]. In **study IV,** salivary levels of total IgA and IgG were higher in SLE patients than in controls. Salivary levels of total IgA and IgG were also higher in SLE-sSS compared to SLE-nonsSS. In **study I,** total IgA and IgG were measured in serum. Interestingly, only the levels of total IgG were higher in the SLE-sSS group (p= 0.009). Furthermore, we observed lower serological IgM levels in the SLE patients as compared to the controls.

In **study IV** we found a negative correlation between the salivary levels of IgG and IgA with serum levels of total IgM, which hss been described in the literature as a provider of protection to autoimmunity. We did not measure total IgM in saliva in the present study, whether the concentrations of total IgM, associate with its counterpart and the other Igs measurements remains unstudied [193,198].

Additional analyses were performed to test total IgA and IgG about subjective and objective oral sicca symptoms. Both total saliva IgA and IgG were higher in the individuals presenting subjective and objective sicca symptoms (data not shown).

Taken together our results confirm a general hyperproduction of immunoglobulins both in plasma and in saliva.

4.2.4 Saliva cytokines and autoantibodies

*4.2.4.1 Cytokines (**study II**)*

In **study II**, CSF-1, TNF-α, IP-10, and MCP-1, as well as calprotectin in saliva were increased in SLE patients as compared to controls ($p<0.05$)**.** Our findings are in line with theories that link the etiology of autoimmunity in SLE and SS to impaired innate immune responses, resulting in high levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IP-10, as a consequence of type I IFNs activation [33,34].

Th-1 lymphocytes are responsible for enhanced production of TNF-α and IFN- γ, both of which induce IP-10 secretion from a variety of cells, perpetuating the autoimmune process, which shifts towards Th2 immunity over the first years after SLE diagnosis, resulting in raised MCP-1 and declining IP-10 concentrations [161]. In **study II,** we observed a simultaneous presence and positive intercorrelations among TNF- α, IP-10, and MCP-1 in saliva. Furthermore, MCP-1, which is upregulated by TNF-α, functions, in part, to regulate the migration and infiltration of monocytes. In accordance, earlier studies showed that these cytokines are involved in the early phases of autoimmune diseases such as SLE and SS [161,199,200], two conditions characterized by type I IFN signature.

In summary, our findings describe interactions between these cytokines, in order to further understand whether a possible co-dependent upregulation exists.

*4.2.4.2 Autoantibodies (**study I** and **IV**)*

The SLE-sSS phenotype is characterized by autoantibodies targeting SSA/Ro and SSB/La antigens, as we demonstrated in **study I,** in line with others [35,45,47-49,55,60]. Analyses of salivary proteins have been suggested to mirror the status of the salivary glands [98] and local production of SSA/SSB autoantibodies within GC- like structures in the salivary glands has been suggested [35,36].

In **study IV,** salivary levels of sub-specificities of ANA were measured in a subset of the participants (SLE n=45 and controls n=36). The levels of anti-SSA/Ro52, anti-SSA/Ro60, and anti-RNP, were higher in SLE patients than in controls. This is in line with earlier studies [42] indicating that autoantibodies are synthesized and secreted within the salivary glands [36,41,201].

In **study I,** 39% of the SLE-sSS subset were seronegative for all anti-SSA/SSB autoantibodies. In **study IV**, the levels in saliva between these individuals (SSA-/SSB-) vs. those presenting SSA and/or SSB antibodies show that even if anti-SSA/SSB are not specific for SS, high levels of anti-SSA/SSB in saliva are almost exclusively present in individuals who are positive for anti-SSA/SSB in serum. Our findings might support possible underlying mechanisms proposed by Dörner and others [41,42,201,202], who suggest that circulating B-cells, and B-cells infiltrating the salivary glands are different, and that memory B-cells are depleted from the circulation and accumulated in the salivary glands of patients with SS. Repeated "mucosal breaks" could drive the initiation of systemic diseases, and increased levels of the B lymphocyte stimulator (BLys, also referred to as B cell-activating factor) detected in the salivary glands may support this theory [202].

We also performed additional analyses investigating whether saliva autoantibodies relate to oral sicca symptoms. Anti-dsDNA and anti-SSA/Ro52 were higher in the individuals presenting subjective and objective sicca symptoms **(Figure 15),** whilst anti-SSA/Ro60 was higher only in individuals with positive objective measurements of oral sicca symptoms **(Figure 15F).**

Taken together, local salivary gland infiltration of leukocytes is presumably responsible for the degradation of the salivary glands, and they could be a possible explanation for our findings in saliva. Moreover, the presence of SSA/SSB autoantibodies in saliva associates with subjective and objective sicca symptoms.

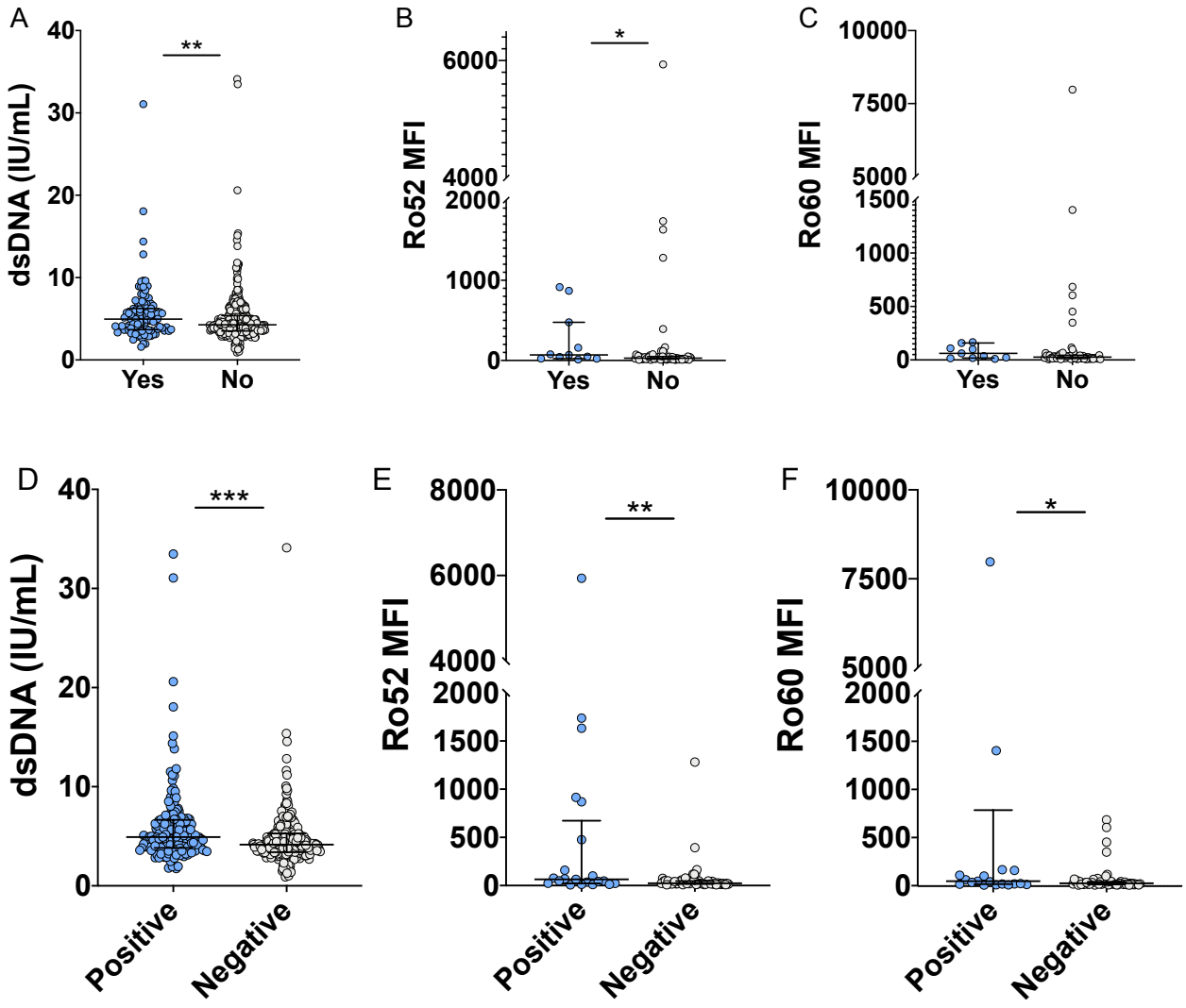


Figure 15. Sicca symptoms measurements:

Subjective; A) levels of anti dsDNA (n=106 *vs*. 365), B) levels of anti-Ro52 (n=11 *vs.* 68) and C) levels of anti-Ro60 (n=11 *vs* 68). **Objective;** D) levels of anti dsDNA (n=168 *vs*. 304); E) levels of anti-Ro52 (n=21 *vs.* 57), and F) levels of anti-Ro60 (n=21 *vs*. 57). Median-IQR. Mann-Whitney test. P = *<0.05, ** <0.01, ***<0,0001.

4.2.5 Correlations among biomarkers in saliva and their counterparts in serum (study II and IV)

In **study II**, correlations between MCP-1 and IP-10 in saliva and their serum counterparts confirm the involvement of these cytokines in SLE and SS in alignment with other studies [161]. In **study IV,** our findings demonstrate that the antibody titers in saliva reflect the serum autoantibody profiles in both SLE patients and controls, and in SLE patients stratified for SS.

Additional analyses were performed in the SLE cohort between the salivary cytokines and autoantibodies measured in the **study II** and **IV** subsets. Of relevance, we found that CSF-1 correlates with all the tested autoantibodies except with anti-dsDNA; and IL-34 correlates negatively with anti-SSA/Ro52 and anti-SSA/Ro60. Finally, TNF-α correlates positively with anti-dsDNA, anti-SSA/Ro52 and, anti-SSB/La **(Figure 16).** An explanation might be that both CSF-1 and IL-34 are related to macrophage homeostasis and induce monocytes to produce TNF-α [159,203]. Our findings strengthen the importance of the innate immunity in SLE (data not shown).

Interestingly, a strong correlation between anti-Rgp IgG and anti-Ro52 was found in the validation subset included in **study III** (**p≤0.05 and rho ≥ 0.5**) (data not shown). This may be explained by an earlier study where they found positive associations between RA, PD, and sicca symptoms as well as with positive lip biopsy [154]. However, we have neither periodontal data from our SLE patients, nor RA patients with sSS to confirm these assumptions.

The autoantibody analyses of **study IV** are still ongoing, the lack of power limits us to draw strong conclusions. Thus far, correlations to disease activity, C3, C4, smoking, and other clinical parameters do not show significant results and do not reflect what other studies have reported [204,205].

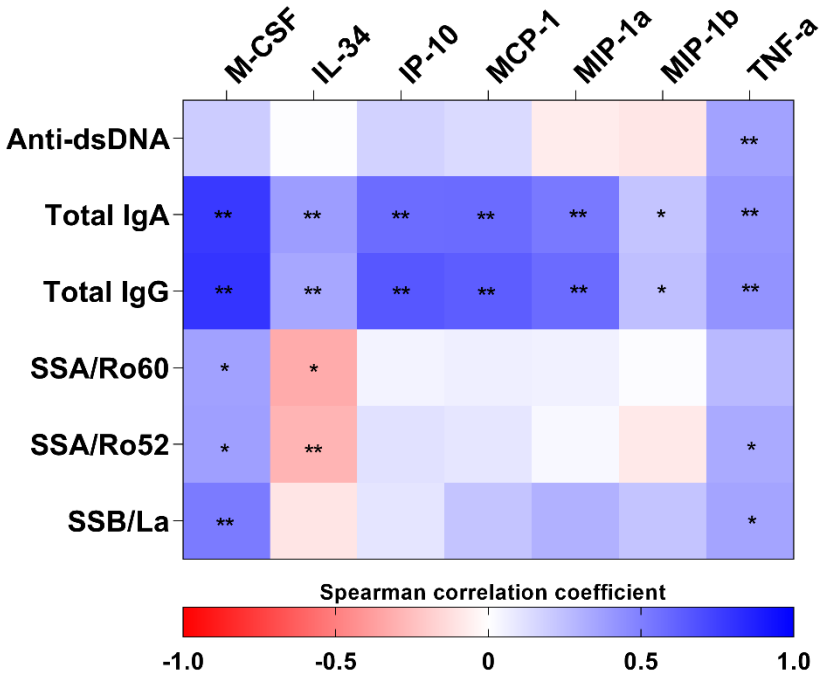
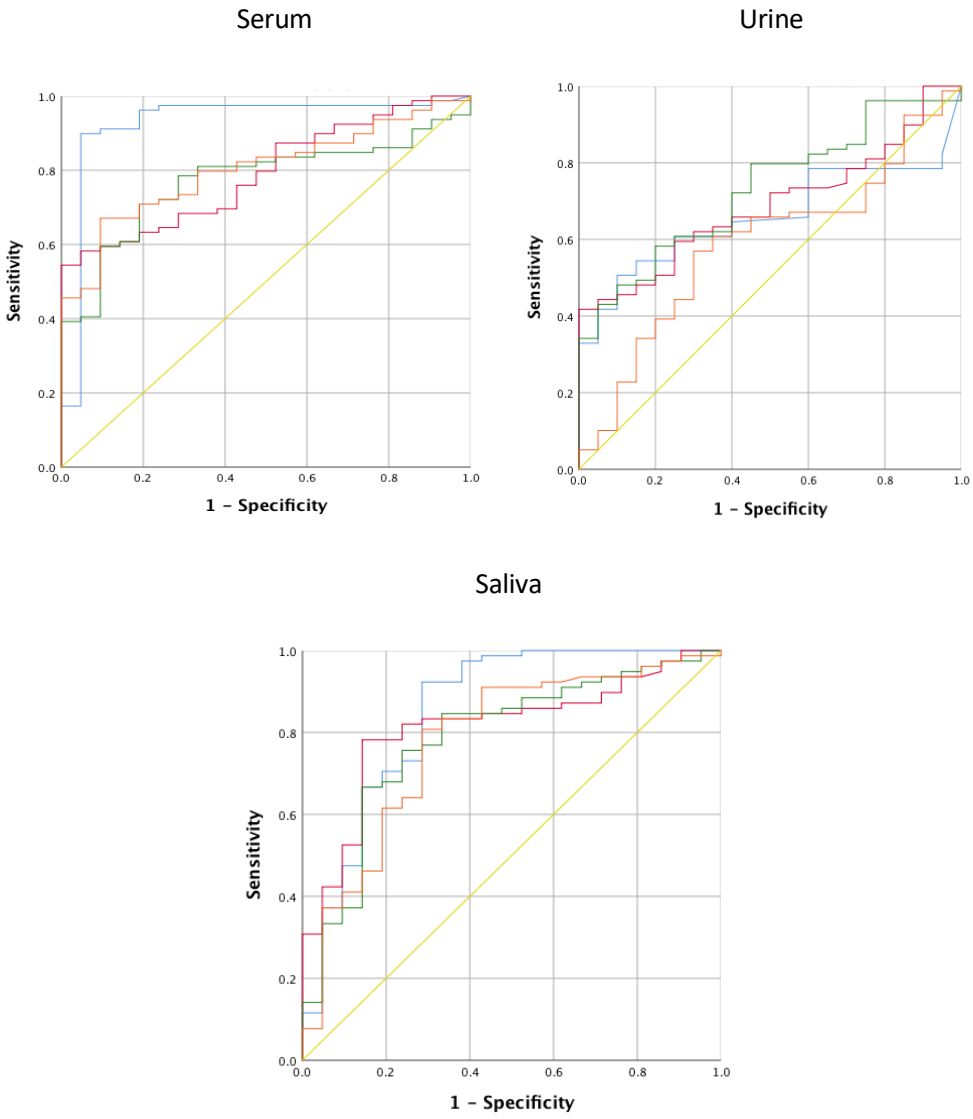


Figure 16. Correlation heatmap of the cytokines and autoantibodies measured in saliva.
Spearman Rho correlation test. P = *<0.05 and ** <0.01, rho ≥ 0.5.

4.3 Ability of saliva to discriminate patients from controls *vs.* serum and urine (study II)

In **study II**, TNF-α, IP-10, and MCP-1 in saliva, serum, and urine, and CSF-1 in saliva and serum distinguished SLE patients from controls. Saliva was demonstrated to perform remarkably well, comparable to as serum, to discriminate patients from controls (area under the curve > 0.764; p<0.001 for all) and saliva outperformed urine for this purpose. TNF-α performed well as a discriminator to separate SLE patients from controls with AUROC of 0.85 in saliva. TNF- α is, thus a potential biomarker. Nonetheless, possible inflammatory confounders such as PD must be explored further [99,116,206].



SERUM	AUC	95% CI	Pvalue	SALIVA	AUC	95%CI	Pvalue	URINE	AUC	95%CI	Pvalue
TNF- α	0.923	0.84 –1.00	<0.001	TNF- α	0.853	0.74– 0.96	<0.001	TNF-α	0.659	0.55– 0.76	0.028
IP-10	0.793	0.70 –0.88	<0.001	IP-10	0.813	0.72 – 0.90	<0.001	IP-10	0.686	0.58 –0.79	0.010
MCP-1	0.773	0.68 –0.87	<0.001	MCP-1	0.791	0.68 –0.90	<0.001	MCP-1	0.732	0.62 –0.84	0.001
CSF-1	0.796	0.71 – 0.88	<0.001	CSF-1	0.764	0.65– 0.88	<0.001	CSF-1	0.586	0.45– 0.71	0.235

Figure 17. Receiver-operating characteristic curves (AUROC). Assessment of the ability of tumor necrosis factor (TNF)- α, interferon-γ-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1 and, colony-stimulating factor (CSF)-1 to discriminate SLE patients from controls. Comparison of serum, saliva, and urine fluids. Each colour represents a marker both in the table and in the curves. Area under the curve with 95 % confidence interval. Reference line in yellow.

4.4 Disease activity and other clinical associations (study I, II, and III)

In **study I,** elevated disease activity, as measured by SLAM (score >6), was more common in the SLE-sSS group (67.2% vs. 57.1%; p=0.05), whereas SLEDAI scores were similar in both groups.

In **study II,** the concentrations of all salivary markers were elevated in patients with high general DA. Earlier studies demonstrated the local production of cyto/chemokines, such as IP-10, in inflamed organs. Further, they suggest the recruitment of mononuclear cells to the damaged site [207]. Taken together, our studies are in line with this hypothesis and local organ damage is a possible explanation for these results. The cellular sources and possible local production of the investigated markers in this study deserve further investigation.

5 Strengths and limitations

5.1 Strengths

A general strength of studies I – V, described in this thesis, is the size and characteristic-distribution of patients. The Karolinska University cohort and the *PAROKRANK* study are large and well-characterized patient groups with their age and sex-matched controls, which gives power and significance to the results presented here. Also, we could investigate a well-characterized periodontitis group in the *PerioGene North* case-control study. Additionally, the use of three different patient groups in **study III** is a strength, where we could investigate our hypotheses in parallel in all three groups.

A novelty presented here is the state of inflammation characterized by pro-inflammatory cytokines presented in the SLE-sSS subset, which is normally defined as a mild form of SLE. Furthermore, to date, no other studies have analyzed potential biomarkers in the three parallel body fluids, saliva, serum, and urine in SLE, as we did in **study II**.

In **study I,** all the patients were tested objectively for sicca symptoms, and similarly the controls with subjective symptoms. To our knowledge, this has not been done previously. Of note, our results showed that subjective symptoms were less common than objective measurement below the SS threshold.

5.2 Limitations

Limitations in our studies; are the potential biases in **study II** since we selected the patients with SLE by the availability of saliva, which might have excluded an important number of patients with sSS.

The levels of pro-inflammatory cytokines in saliva are unspecific and could have been affected by the presence of oral conditions, e.g., periodontitis. We don't have investigations of the oral status in the SLE cohort which limits several of the comparisons in **study III.**

Also, regarding the oral status, a weakness was the lack of standardization in the definitions of periodontitis in **study III**, where differences in the two periodontitis studies *PAROKRANK* and *PerioGene North* were present. A new classification of periodontitis [131] was recently launched, and it indicates that some positive periodontitis might have been disregarded by diagnosing solely based on Panorama x-rays.

Moreover, in **study IV** the laboratory analyses are incomplete due to a problem with the delivery of the ELISA kits, the small study sample in the present manuscript limits us to perform comparisons and draw conclusions, e.g., with salivary total IgM, or as is the case in **study II,** with the presence of oral ulcers.

We considered the differences in the salivary flow and *total protein* of the SLE with and without SS as a potential weakness, nonetheless significant results remained after adjustments for salivary flow.

6 Concluding remarks

- Strictly applying the 2002 American-European Consensus Criteria (AECC), secondary-Sjögrens syndrome (sSS) affects a quarter of the systemic lupus erythematosus (SLE) patients, the frequency of sSS increases with increasing age.

- Despite not being included in the 2002 AECC, SSA/SSB autoantibodies are overrepresented in the SLE-sSS subset.

- The inflammatory state demonstrated in the SLE-sSS *vs.* SLE-nonsSS, as expressed with elevated pro-inflammatory cytokines, may have a therapeutic potential.

- Novel innate immune-related biomarkers in saliva offer a non-invasive alternative to serum to monitor SLE disease activity.

- Antibodies to *P. gingivalis,* Rgp IgG associated with periodontitis severity and the occurrence of ACPA and dsDNA antibodies, which suggests an implicative role of anti-Rgp IgG as an indicator for increased risk of SLE- and rheumatoid arthritis (RA)-related autoimmunity in periodontitis patients.

- The occurrence of the SLE and RA autoantibodies; aPL, ACPA, and anti-dsDNA in periodontitis and MI strengthen possible associations between periodontitis and autoimmunity.

- Antibody measurements in saliva reflect the serum autoantibody profiles in SLE with and without sSS. Anti-SSA and -SSB have a strong relationship with SICCA symptoms in both serum and saliva.

- The oral cavity may help to identify novel pathways to autoimmunity and provides us with alternatives to explore biomarkers with similar “accuracy” as serum and urine.

7 Future perspectives

Our long-term goal is to evaluate if salivary biomarkers, including autoantibodies, can be used to diagnose, monitor, or predict sSS or other autoimmune features of SLE.

The presence of elevated pro-inflammatory cytokines, the autoantibodies found in saliva, in the above works, should be studied in earlier stages of SLE to determine whether they can predict the onset of SS.

A standardized classification criterion for periodontitis for research purposes should be identified to avoid discrepancies in the investigations and comparisons since the clinical diagnosis has recently changed. Our patients were classified with the old criteria, and therefore there is a risk that our findings lose impact when the new classification becomes more established.

Future studies are needed to investigate whether salivary levels reflect the grade of degradation or inflammation in the salivary glands by correlating with the levels of these markers to salivary glands biopsies.

From a general clinical perspective, the early detection of patients prone to develop Sjögrens syndrome could avoid unnecessary oral-cavity-related morbidities. And perhaps, with better treatments in the future, the development of Sjögrens syndrome altogether.

Further analyses will be performed to confirm our preliminary findings in the pilot **study IV** with follow-up data.

8 Acknowledgements

”Yo vengo de todo el mundo, vengo de toda la gente…” Facundo Cabral, 1937-2011.

I would like to thank the following people, without whom I would not have been able to complete this thesis, all of you have been a part of this journey. Thank you for the advice, help, support, and love.

First, I want to thank God, the Universe, Nature, and/or the Tao, names are not definitions. For all that I am, what I have, and what I don't have. If I am something, it is because of you.

A La maestra Maria Elena, por alentar mi creatividad y autonomía. Esta tesis no sería posible si no hubiése sido alentado a trabajar a mi modo y a mi ritmo en mis primeros años de escuela. Gracias!

Stor TACK till Elisabet Svenungsson för att du har bjudit in mig till att börja denna resa och blir Medicine doktor. Jag är jättetacksam för din kunskap, ditt engagemang, tålamod och för att du introducerade mig i den fantastiska och intressanta världen av autoimmuna sjukdomar.

Till Iva Gunnarsson, för att du gav mig jobbet! Det var tack vare dig allt började. Elisabet och du har varit de bästa cheferna jag någonsin haft. Iva, du är en inspiration! Tack till Linn också, för att hon presenterade mig för dig. Till Elias och Gustaf, mina första FileMaker- och labbpartners! Till Vera, som tog över de uppgifterna.

Till Elisabeth Boström min bihandledare, för att du öppnade dörrarna till ditt labb och introducerade mig till din grupp. En stor del av mina doktorandstudier var där. Tack för att du och Elisabet har varit så stöttande dessa sista dagar och tagit av er privata tid för att hjälpa mig att få ihop kappan.

Till de svenska skattebetalarna, and the different sources of my financial support for generous grants, to the patients for believing in our research. To our collaborators from the *PAROKRANK* group: Lars Rydén, Barbro Kjellström, Björn Klinge, Anders Gustafsson, and Kåre Buhlin. To our collaborators from the *PerioGeneNorth* study: Pernilla Lundberg and Elin Kindstedt. Tack båda grupper för att jag fick lära mig ifrån Er!

To all the people from the Rheumatology unit, for each of you have been a source of inspiration. Ioannis, Isak, and all the people from Doktorandklubben, former and current, thank you for your kind advice and constructive feedback, as well as for the nice company during the conferences we attended together. To my co-authors for providing me with unvaluable input. To Vilija and Giorgia, the only two people I know (besides Elisabet) who uses JMP, thank you for helping me discover that tool. Till min medförfattare Marika Kvarnström, som har lärt mig allt hon kan om Sjögrens syndrom och för all hjälp med att granska den delen av kappan som handlar just om det.

Thanks to Laurent Arnaud, who helped me write my first grant application. You gave me the best advice regarding an application, and it will always stay in my head. To the people at my office, Yogan you have been very helpful these last few days. Fabricio, gracias por las múltiples discusiones sobre reumatología, estadística, las consultas médicas, y por ponerle los puntos a las íes en mi kappa. Karen, Joakim, Henrik, Kristoffer, Shahrzad, och Susanne, kontoret var alltid bättre när Ni fanns där. And to those who were there in the past, Valery for starting the SPSS journey with me, Kristina for helping me to put together my documents, and the new member Aliisa, I acknowledge you for the acknowledgments.

Till Gunnel, Sirpa, Veronica, Stina, Sanna, Helena, Lillemor och Michael, tack för att ni underlättade all administration och gjorde att allt flöt smidigt. Till Sonia, tack för att du hjälpte mig att hitta data som jag inte kunde hitta i FileMaker och för att hitta patienter till den nya salivstudien. A Karin y Nancy gracias por el círculo de latinos y bailaores que han creado. Jelena, when are you back? Timmy and Magnus my closests IT advisors -restart and turn off- the key to many doors…

The people at CMM, for letting me invade your space, your labs, and your groups. For letting me learn with you and from you. For the philosophy, all the fika, and the CMM pubs together. Angeles, al parecer hemos compartido bastante, el TOEFL, GraphPad, mudanzas y hasta un que otro tequila, gracias! A Esteban (Sebastián), gracias por ayudarme a organizar mi data, y por el “yogurt time”. Begum, for showing me Youden's Index. Lina, for trying to teach me to make cluster analyses in R.

Evelyn, Boris, Randy, Anthony, Natalie, Lorenzo, Henna, Agnes, and many others: thank you for all our moments together. To all the organizers of the CMM pubs, thank you for the joy you brought and the cheap beers. To DJ Jorge, gracias por la buena música, y por la no tan buena también. Maria, Lewandowsky, and all the others on the 5th floor, on other floors and in other labs, thank you. Christina, gracias por siempre tener palabras positivas. Karin Ch., thank you for being available to answer my "quick questions".

To Charlotte, my coauthor, I’m grateful for the collaboration. For letting me work at my own pace and understanding my absence during the rush times at the clinic. A special acknowledgment to Leonid, it was with you that I started my contact with the lab in Sweden. I remember your words of encouragement, even though I had just made a mistake. Please say thanks to your son, who was the best lab companion that summer. To Emelie and Marc for guiding me during the HLA typing.

Till Karin Lundberg, jag är väldigt tacksam och varje dag imponeras jag mer av all den kunskap du besitter och är villig att dela med dig av. Jag är tacksam för att du först var med i min halvtidskommitté och sedan samarbetade med mig. Tack också för att du granskade RA-delen på min kappa. Gloria, muchas gracias por la ayuda con las muestras en el lab. Tack till Eva J. som lärde mig att ha en bra ordning och struktur på mina prover, till Julia som alltid var villig att hjälpa mig att hitta prover.

Ronaldo my friend, coworker, and co-author, for explaining to me and helping me with patience, and for always improving my figures. Mirjam, thank you for forcing me to run my first ELISA, and to Daniela for not letting me run it. Niki, my lab manager in the Dental Faculty, thank you for taking care of my orders when I was away. To all the people at DentMed/ANA Futura, those who are still there and those who already left, for all the nice moments together. A Manuel Patarroyo, gracias por alentarme cuando apenas estaba preparando mis pruebas y enseñarme la importancia de las cosas aparentemente simples y pequeñas. To Taichi, Robert, Reuben, Natalija, Tuläy, Anna, and all the others, you know who you are. Thanks for all your support! Dave, thank you for "killing" my English.

To the members of my half-time committee, that I haven't mentioned yet, Karin Garming-Legert and Roland Jonsson for the outstanding input and advice as well as for motivating me to continue with my projects. Till mina mentorer Erik och Patricia, tack för att ni har varit där för mig, för de goda råden och för att visa mig vägen tillbaka till den kliniska aspekten av mina studier.

Till FTV Sörmland där jag startade min forskning, till Lars, mina kliniska handledare på Käkkirurgiska avdelningen MSE, till Patrick, Magnus, mina chefer och mina sköterskor för att se till att det alltid fanns forskningstid i mitt schema och för er handledning. Till mina kollegor på STV Eskilstuna och STV Nyköping, till Anna, Sibylle, Paula, Lillemor, Madde. För att ni har varit ett underbart team. Att kunna ha ett bra team hjälpte alltid mina doktorandstudier. Till FoU Sörmland, till Peter V., Leonardo för att ni stötte mina projekt.

Till FTV Stockholm, min kliniska handledare på Eastman Institutet Björn Johansson och till Carina Kruger for att ni välkomnade mig i den käkkirurgiska avdelningen. Till FoU avdelning, Lars, Pia, och till journalklubben. Till Eva, Nomi, Elnaz, FTV Nynäshamn, FTV Gustavsberg, FTV Fruängen, FTV Sickla, FTV Tyresö, för att Ni har varit så bra kollegor. Ritva för du är bäst! Till Israt, Camilla, Stina, Francis, Tiba, Hanna, Maja, Jeanette, tack!

To Madeleine, Moa, Kristina. Danuta, for the support in the heavy times you were by my side. To Aylin for the good chats and beers, to Rebecka for the writing tips, Dmitry for the support during the writing of my first paper, thank you for helping me to structure it! To Gregory, what can I say? … You could have helped me more, so I had something to say... Just kidding my friend, seeing your dedication inspired me to take the decision of becoming a Ph.D. student. Although you always tried to discourage me with your words, your actions weighed more.

Till familjen Ricknert, för att ni var min familj i Sverige under en lång väg av denna resa. Louise, du var en otrolig bra partner under den tiden du var vid min sida och för det kommer jag alltid bli tacksam för. Till Rune†, Gunvor†, Ingrid† som inte är i denna värld längre men jag har deras ord med mig varje

dag. Till Olof och Eva, Susanna och familj, Caroline och familj. Tack för att Ni alltid hade bara peppande ord.

To Chelas y to La Neta, pobres de ustedes que tenían que cargar con mis malos ratos. Gracias Amelia, por echarle un ojo a los agradecimientos. A mis Mexicanos de el grupo de Nyköping (incluye los de Estocolmo). A Martha, Aline, Paola, Rodrigo. To those who have left, Margarita, Alina, Magdalena, Ella, Diana, Raúl, Mayela, I know you tried. Muchas gracias por su amistad, por aguantar que llegará tarde con las papas, o que confirmará a última hora, etc.

A Pablo Galindo uno de mis mentores y una gran inspiración para mi, gracias también a todo el equipo a su alrededor durante mi estancia en 2008. No cabe duda que su trabajo dejó huella en mi y me alentó a terminar éste viaje. A México y a los Mexicanos que pagaron con sus impuestos mi educación, espero poder devolver a México algo de lo que me dió. A mis maestros y mentores de la escuela de Odontología; al Dr. Carrillo que siempre vió más potencial en mí y me insistía en sacarlo. Al Dr Bueno, al Dr. Arriola y a la Dra Gallegos que fueron una pieza importante para que yo pudiera estar aquí.

A mis “familias adoptivas” especialmente a Doña Dora y Don Héctor, por los consejos, por los regaños y los jalones de oreja. Por supuesto a Hugo y a Luis. Yvonne; que puedo decirte que abarque todo lo que puedo agradecerte, con fines de educación te agradezco el siempre alentarme a sacar todo mi potencial.

A Eugenia Ruacho, a la abuela Angélica, a la niña María y a la bebé Alejandra, vaya familia! Jenny thank you for being there when you were needed and not being there when you weren’t needed. Thank you for helping me with the figures, your sense of aesthetics was crucial in the editions.

Finalmente, a mi familia, padres y hermanos. Papá, gracias por todo! Por enseñarme responsabilidad y respeto, a trabajar y ganarme lo que yo quiera con mi esfuerzo, de ti aprendí con el ejemplo y no con palabras. Mamá, gracias por la vida, por regalármela primero y después darme la libertad para vivirla a mi modo. Sé que no fue fácil dejar ir a tu hijo de 14 años a vivir, primero a otra ciudad, después temporalmente a otro país y ahora indefinidamente. Nadie puede dar algo más valioso que éso. A mis hermanos mayores; Lupita, César, Olivia Y Karina porque gracias a ustedes aprendí la responsabilidad, siempre los vi y los seguiré viendo con respeto y como un ejemplo. Por último, a mis hermanos menores Javi y Carmen por hacerme ver que siempre hay alguien que nos mira y nos admira y que no podemos decepcionar; espero y aun no sea tarde para no defraudarlos.

Please, if I forget someone forgive me! Those who have already gone through this will understand, and those who haven't yet will understand soon...

Thank you all, this thesis is also yours!

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