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IMMUNOPATHOGENIC MECHANISMS IN PRIMARY SJÖGREN'S SYNDROME

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Immunopathogenic mechanisms in primary Sjögren's syndrome

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

Primary Sjögren's syndrome is a systemic autoimmune disease primarily affecting salivary and lacrimal glands, but patients may also have other organ involvement. Development of Sjögren's syndrome has been associated with genetic polymorphisms and aberrances in innate and adaptive immunity, which include abnormal B cell functions with autoantibody production, and increased activity of the interferon (IFN) system. Microbial triggers have been suspected to partake in the pathogenesis. The overall aim of this thesis was to characterize exogenous and endogenous mechanisms contributing to the immunopathology of Sjögren's syndrome.

Genetic variants associated with Sjögren's syndrome convey low risk-increase for disease development, indicating that environmental factors must play an important role. However, at the start of the work leading up to this thesis, no environmental risk factors for Sjögren's syndrome had been identified. Therefore, to assess the influence of smoking, we performed the largest questionnaire-based case-control study to date. We found that current and ever smoking was less frequent among patients compared to controls, however, smoking patterns were similar up until approximately 35 years before diagnosis and smoking thereafter declined in patients. We conclude that smoking does not appear to be a risk factor for development of Sjögren's syndrome, but that early symptoms of dryness may influence behavior. Further, to define connections between previous infections and disease development, we extracted data on patients and matched controls from the Swedish National Patient Register. We found that a history of infection was associated with a higher risk of Sjögren's syndrome, with overall stronger associations to anti-Ro/SSA and anti-La/SSB positive disease. Infections of the respiratory tract associated both with autoantibody positive and negative disease, and a dose-response relationship was present in autoantibody positive patients.

Previous studies have identified *CXCR5* as a susceptibility locus for Sjögren's syndrome. We describe a novel eQTL effect for *CXCR5* in B cells. Further, decreased percentages of $CXCR5^+$ cells with lower CXCR5 surface expression in the circulation coincided with higher CXCL13 plasma protein levels, and higher numbers of $CXCR5^+$ cells were detected in salivary glands of patients compared to controls. We conclude that $CXCR5^+$ cells likely relocate from the blood stream to the autoimmune target tissues in patients. By transcriptomic analysis of peripheral B cells, we found prominent type I and type II IFN signatures, as well as higher expression of chemokines and chemokine receptors in patients compared to controls, while inhibitors of cytokine signaling were downregulated. Our data add to the understanding of B cells in Sjögren's syndrome and define potential candidates for future functional studies.

Activation of the IFN system is often assessed by quantifying the expression of IFN regulated genes. However, RNA samples are not always available, wherefore alternative methods are needed. We describe two novel IFN scores calculated from protein levels in serum or plasma, and from levels of DNA methylation.

Responses to microbial antigens can be studied *in vivo* during vaccination. We therefore assessed serological and cellular responses to influenza vaccination in patients with Sjögren's syndrome and SLE. Untreated patients with Sjögren's syndrome and SLE patients receiving no or light treatment responded to the viral antigens with higher vaccine-specific antibody titers compared to controls. Augmented antibody responses were associated with upregulated markers of type I IFN system activation at the mRNA level in monocytes and at the protein level in the circulation before vaccination. Specific components associated with a higher serological response were identified, which warrant further study. Our data increase the comprehension of immune reactions toward microbial antigens in autoimmune disease.

In conclusion, this thesis expands our current understanding of environmental factors and immunopathogenic processes in primary Sjögren's syndrome.

LIST OF SCIENTIFIC PAPERS

I. **Cigarette smoking patterns preceding primary Sjögren's syndrome**
Mofors J*, Björk A*, Richardsdotter Andersson E, Kvarnström M, Forsblad d'Elia H, Magnusson-Bucher S, Padyukov L, Kockum I, Hillert J, Eriksson P, Mandl T, Nordmark G, Alfredsson L, Wahren-Herlenius M
RMD Open. 2020 Sep;6(3):e001402

II. **Infections increase the risk of developing Sjögren's syndrome**
Mofors J, Arkema EV, Björk A, Westermark L, Kvarnström M, Forsblad-d'Elia H, Magnusson Bucher S, Eriksson P, Mandl T, Nordmark G, Wahren-Herlenius M
J Intern Med. 2019 Jun;285(6):670-680

III. **Viral antigens elicit augmented immune responses in primary Sjögren's syndrome**
Björk A, Thorlacius GE, Mofors J, Richardsdotter Andersson E, Ivanchenko M, Tingström J, James T, Brokstad KA, Cox RJ, Jonsson R, Kvarnström M, Wahren-Herlenius M
Rheumatology (Oxford). 2020 Jul 1;59(7):1651-1661

IV. **Interferon activation status underlies higher antibody response to viral antigens in patients with systemic lupus erythematosus receiving no or light treatment**
Björk A, Da Silva Rodrigues R, Richardsdotter Andersson E, Ramírez Sepúlveda JI, Mofors J, Kvarnström M, Oke V, Svenungsson E, Gunnarsson I, Wahren-Herlenius M
Rheumatology (Oxford). 2020 Oct 2:keaa611.

V. **Protein and DNA methylation-based scores as surrogate markers for interferon system activation in patients with primary Sjögren's syndrome**
Björk A, Richardsdotter Andersson E, Imgenberg-Kreuz J, Thorlacius GE, Mofors J, Syvänen AC, Kvarnström M, Nordmark G, Wahren-Herlenius M
RMD Open. 2020 Jan;6(1):e000995

VI. **Transcription profiling of peripheral B cells in antibody-positive primary Sjögren's syndrome reveals upregulated expression of CX3CR1 and a type I and type II interferon signature**
Imgenberg-Kreuz J, Sandling JK, Björk A, Nordlund J, Kvarnström M, Eloranta ML, Rönnblom L, Wahren-Herlenius M, Syvänen AC, Nordmark G
Scand J Immunol. 2018 May;87(5):e12662

VII. **Diminished CXCR5 expression in peripheral blood of patients with Sjögren's syndrome may relate to both genotype and salivary gland homing**
Aqrawi LA, Ivanchenko M, Björk A, Ramírez Sepúlveda JI, Imgenberg-Kreuz J, Kvarnström M, Haselmayer P, Jensen JL, Nordmark G, Chemin K, Skarstein K, Wahren-Herlenius M
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*Equal contribution

RELATED PAPERS NOT INCLUDED IN THE THESIS

Environmental factors in the pathogenesis of primary Sjögren's syndrome
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CONTENTS

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
AECG	American-European Consensus Group
APS-1	Autoimmune polyendocrinopathy syndrome type 1
BAFF	B cell activating factor
BICLA	British Isles Lupus Assessment Group-based Composite Lupus Assessment
CAAR	Chimeric autoantibody receptor
CCL	CC motif chemokine ligand
CCR	CC chemokine receptor
CXCL	CXC motif chemokine ligand
CXCR	CXC chemokine receptor
DAMP	Damage-associated molecular pattern
DMARD	Disease-modifying anti-rheumatic drug
DNAm	DNA methylation
EBV	Epstein Barr-virus
EGM	Extra glandular manifestation
eQTL	Expression quantitative trait loci
ESSDAI	EULAR Sjögren's Syndrome Disease Activity Index
ESSPRI	EULAR Sjögren's Syndrome Patient Reported Index
EULAR	European League Against Rheumatism
FDC	Follicular dendritic cell
FOXP3	Forkhead box P3
GC	Germinal center
GWAS	Genome-wide association studies
HCQ	Hydroxychloroquine
HLA	Human leukocyte antigen
HTLV-1	Human T-lymphocyte virus type 1
ICD	International Classification of Diseases
IFN	Interferon
IFNAR	Interferon-a/b receptor
IKK	Inhibitor of nuclear factor-kappa B kinase
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
IκB	Inhibitor of nuclear factor-kappa B

IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK1	Janus kinase 1
La/SSB	Sjögren's-syndrome-related antigen B
MALT	Mucosa-associated lymphoid tissue
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MZ	Marginal zone
NEMO	Nuclear factor-kappa B essential modulator
NF-κB	Nuclear factor-kappa B
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PEA	Proximity extension assay
pIFN	Protein interferon
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
Ro/SSA	Sjögren's-syndrome-related antigen A
SGEC	Salivary gland epithelial cell
SLE	Systemic lupus erythematosus
SLEDAI	SLE Disease Activity Index
SNP	Single nucleotide polymorphism
SOCS	Suppressors of cytokine signaling
STAT	Signal transducer and activator of transcription
Tfh	T follicular helper cell
Th	T helper cell
TLR	Toll-like receptor
Treg	Regulatory T cell
TYK2	Tyrosine kinase 2
UC	Ulcerative colitis

1 INTRODUCTION

Autoimmune diseases are characterized by a loss of self-tolerance, which results in autoreactive immune cells and autoantibodies that may inflict tissue damage and cause inflammation. The importance of a detailed understanding of underlying immunopathogenic mechanisms in these diseases is evident though the implementation of biologics - predominantly monoclonal antibody-based drugs specifically targeting certain parts of the immune system. Moreover, measures to prevent autoimmune diseases rely on the identification of environmental risk factors that trigger or propagate disease.

This thesis will focus on mechanisms involved in the autoimmune pathology of primary Sjögren's syndrome, and to some extent systemic lupus erythematosus (SLE).

1.1 THE IMMUNE SYSTEM

The immune system has evolved to protect the body against harmful pathogens and to maintain homeostasis. It is commonly divided in two functional parts: innate immunity which provides fast but less specific protection, and adaptive immunity which acts slower in primary infections but can generate highly specific and effective responses. As implied by the name, innate immunity depends on inherited receptors for recognition of pathogens or other danger signals, whereas the adaptive immune system has the ability to generate specialized recognition receptors depending on which antigens that are encountered. Innate and adaptive immunity are highly interconnected through an intricate network of tissues, cells, and signaling molecules. Some aspects of the immune system of particular relevance for this thesis will be briefly reviewed below.

1.1.1 Innate immunity

Innate immunity constitutes a first-line defense and comprises barriers such as the skin and mucosal tissues as well as anti-microbial molecules therein, plasma proteins including the complement system, and cells in the circulation and in tissues. Innate immune cells have so called pattern recognition receptors (PRRs) which enable detection of microbial molecules and endogenous signs of damage, usually referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively. Upon recognition of such patterns, cells of innate immunity may perform phagocytosis, kill infected cells, and/or promote inflammation and recruitment of adaptive immunity. The PRRs can be classified into five major families, of which the toll-like receptors (TLRs) are or special relevance for this thesis. Another important feature of the innate immune system is the ability to secrete anti-microbial interferons (IFN), which are implicated in several systemic autoimmune diseases. Whereas the involvement of adaptive immunity to autoimmune disease is obvious through autoreactive T and B cells, innate immunity also plays important roles, which I together with colleagues have reviewed in [1].

1.1.1.1 Toll-like receptors

Toll-like receptors are important PRRs which recognize a variety of conserved microbial components. The Toll protein was first described in *Drosophila melanogaster* where it was shown to play a role in embryonic development [2], but was later recognized also for its importance in protecting the flies against infections [3]. As of today, ten receptors (TLR1-10) have been described in humans, which are either present in the plasma membrane, or in endosomes (TLR3, 7-9) where they can recognize nucleic acids. Upon TLR ligation, downstream signaling results in activation of transcription factors, including members of the nuclear factor-κB (NF-κB) protein family, and the endosomal TLRs as well as TLR4 can induce production of type I IFNs.

1.1.1.2 NF-κB signaling

Nuclear factor-κB is a protein complex of intracellular transcription mediators which are of fundamental importance for inflammatory responses as well as for regulation of innate and adaptive immune processes.

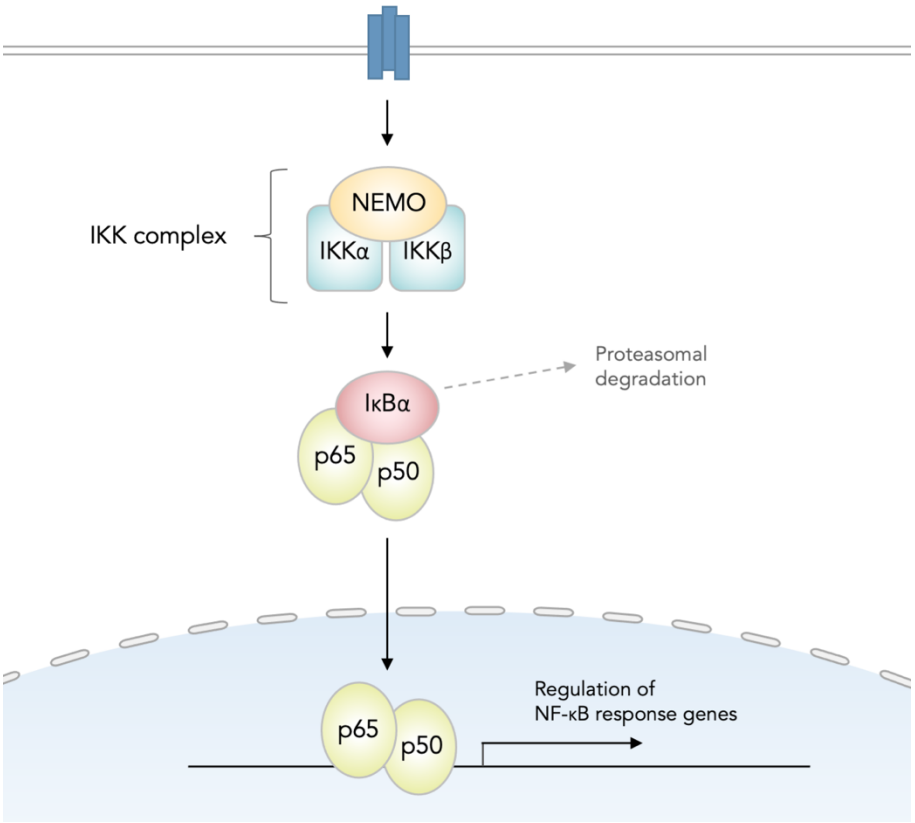

***Figure 1**. Schematic overview of classical NF-κB pathway signaling.*

Beyond induction of numerous inflammatory genes including those encoding cytokines and chemokines, the NF-κB family also regulates activation, differentiation and survival of immune cells [4]. The family consists of five proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), which form various homo- and hetero-dimers, but all exert their function by binding a sequence motif called κB enhancer [4]. Prior to activation, such protein dimers are found in the cytosol in a suppressed state. The suppression is mediated by a family

of inhibitors denoted Inhibitors of nuclear factor-κB (IκBs), with the most important member being IκBα. Simplified, classical activation of NF-κB is achieved through phosphorylation followed by ubiquitination and proteasomal degradation of IκBs by the IκB kinase (IKK). The IKK consists of a heterodimer of IKKα and IKKβ and a regulatory protein called NF-κB essential modulator (NEMO). Upon degradation of the IκBs, the NF-κB protein dimers enter the nucleus to induce gene expression (**Figure 1**).

1.1.1.3 The interferon system

The anti-viral properties of IFNs have been known since their initial discovery in the 1950s [5, 6]. In humans, the IFN family consists of type I, II, and III IFNs, which are classified according to which receptor they bind. The type I IFNs are the largest class consisting of 12 subtypes of IFN-α encoded by 13 genes, IFN-β, IFN-ε, IFN-κ, and IFN-ω. All type I IFNs bind and signal through the ubiquitously expressed interferon-α/β receptor (IFNAR) which is a heterodimer of the subunits IFNAR1 and IFNAR2 [7]. Upon binding, the receptor subunits dimerize leading to activation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which, in canonical type I IFN signaling, causes receptor phosphorylation and recruitment and phosphorylation of signal transducer and activator of transcription (STAT) proteins (**Figure 2**). Simplified, heterodimers of phosphorylated STAT1 and STAT2 form a complex with IFN regulatory factor (IRF)9 denoted interferon-stimulated gene factor 3 (ISGF3) which binds and induces expression at IFN-stimulated response elements (ISRE) sites (**Figure 2**). However, signaling via other combinations of STATs as well as signaling not mediated via the JAK-STAT pathway also occurs [8].

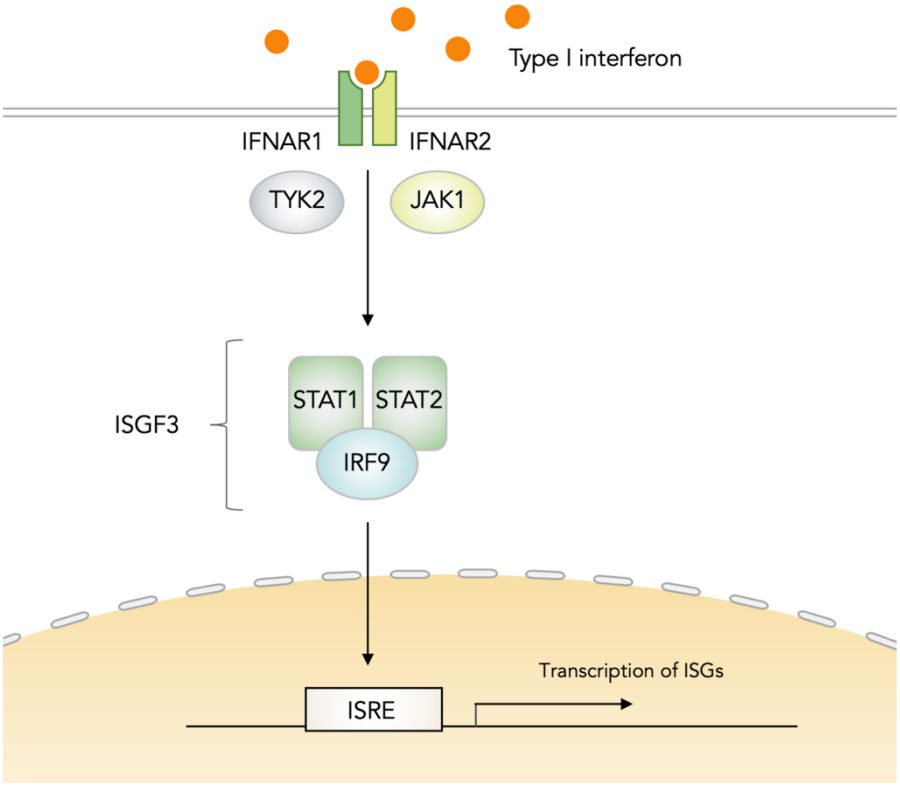

***Figure 2**. Schematic overview of canonical type I interferon pathway signaling.*

Type II IFN consists solely of IFN-γ which has modest anti-viral activity but is important for activation of macrophages and for governing differentiation of T cells [9]. The type III IFNs are the most recently described and least studied group consisting of IFN-λ1 (IL29), IFN-λ2 (IL28A) and IFN-λ3 (IL28B). Type III IFNs elicit responses similar to type I IFNs, but signal through another receptor with more limited tissue expression, primarily on epithelial cells [10].

1.1.2 Adaptive immunity

Adaptive immunity is phylogenetically younger and more complex compared to innate immunity. T and B cells are central to the functions of adaptive immunity and both carry epitope-specific receptors denoted T and B cell receptors, respectively. Each cell carries receptors recognizing only one antigen, which had been hypothesized in Burnet's clonal selection theory more than 80 years ago [11]. The B cell receptor constitutes a membrane-bound immunoglobulin which may be secreted as antibodies, whereas the T cell receptor is always membrane bound. B cell receptors can recognize various types of molecules, whereas T cell receptors will only recognize peptide antigens displayed by major histocompatibility complex (MHC) I or MHC II molecules. In contrast to the germline encoded receptors of innate immunity, cells of the adaptive immune system have the capacity to modify their receptors through DNA rearrangements [12]. An astonishing variety of receptor specificities is achieved through stochastic rearrangement of various V, D and J segments in the immunoglobulin and T cell receptor genes. B cells can further adapt and perfect their receptors through isotype switching, somatic hypermutation, and affinity maturation by interaction with T cells in peripheral lymphoid organs.

The chemokine receptors CXCR5 and CCR7 promote migration of T and B cells into peripheral lymphoid organs in which some cells participate in the formation of germinal centers (GCs), where high-affinity antibody-secreting cells are generated from B cells with the help of specialized follicular dendritic cells (FDCs) and T follicular helper cells (Tfh). B cells achieving the highest affinity will then differentiate into memory B cells and long-lived plasma cells, whereby an immunological memory is generated.

1.1.2.1 Tolerance

In a healthy state, adaptive immunity thus harbors the ability to target virtually any antigens that may pose a danger, but simultaneously retains the ability to avoid attacking antigens from the own body. This discrimination between self and non-self is denoted immune tolerance and errors during this process may result in autoimmune disease.

During the 1940s and 50s, it became evident that immune tolerance must be established during early life. It had been observed that dizygotic twin cattle sharing a common circulation through the placenta became tolerant of the other twin's blood cells later in life [13]. Further, experiments in mice showed that inoculation of cells from another donor during fetal life made the recipient tolerant to skin grafts from the donor even during adult life [14]. These observations eventually led to the hypothesis that self-reactive lymphocytes are removed during maturation [11], termed clonal deletion.

Immune tolerance is broadly divided into central and peripheral tolerance mechanisms. Central tolerance mechanisms refer to negative selection of T and B cells in cases where somatic recombination has resulted in high receptor affinity for antigens presented in the thymus or bone marrow, and also to the generation of immunosuppressive regulatory T cells (Tregs). Nevertheless, B cells that recognize self-epitopes may undergo an additional process named receptor editing to become non-self-reactive B cells. During maturation in the thymus, T cell are presented with self-antigens from all tissues of the body by virtue of the transcription factor *AIRE*, which promotes the expression of proteins not otherwise expressed in the thymus [15]. Mutations in the *AIRE* gene cause the autoimmune condition autoimmune polyendocrinopathy syndrome type 1 (APS-1) in humans [16, 17], further underlining the importance of this gene during tolerogenic processes.

Autoreactive lymphocytes may however escape central tolerance mechanisms whereby peripheral tolerance mechanisms can act to prevent damage. Such mechanisms include immune suppression by Tregs, cell death or anergy by antigen recognition without co-stimulation, or by expression of death receptors such as Fas and Fas ligand. The *forkhead box P3* (*FOXP3)* gene is essential for development and function of Tregs, and mutations in this gene results in the autoimmune immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome [18]. Furthermore, inhibitory T cell receptors such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are also important for peripheral tolerance mechanisms. Indeed, monoclonal antibodies targeted at these receptors, which have been developed for treatment of cancers, may, as an adverse effect, induce autoimmune reactions in humans [19].

In all, numerous mechanisms orchestrate a delicate balance between protective immunity and self-tolerance, in which failure of tolerogenic mechanisms may result in development of autoimmune disease.

1.2 AUTOIMMUNE DISEASE

Autoimmune disease thus results from an erroneous targeting of the immune system against healthy tissue. The concept of autoimmunity was first established in 1900 by immunologist Paul Ehrlich who coined the term "*horror autotoxicus*" to describe the unwillingness of the immune system to target the own body [20]. Ehrlich postulated that autoimmunity was not possible, and due to his substantial influence in the field of immunology the existence of such diseases remained controversial for a long time. Starting in the 1950s, a modern understanding of autoimmunity and autoantibodies formed [21]. Whereas there is still disagreement regarding which diagnoses that meet the criteria to be classified as an autoimmune disease, community prevalence has been estimated at around 4.5 to 9.4% [22, 23]. Apart from the significant morbidity associated with the diseases, much-increased mortality rates are observed [24, 25]. Data on the overall cost of the burden of autoimmune disease are scarce, but due to the chronic nature of the diseases and a high prevalence of co-morbidities the societal costs are undoubtedly substantial.

Although great advances have been made regarding treatment, autoimmune diseases generally cannot be cured. Traditionally, corticosteroids in combination with disease-

modifying anti-rheumatic drugs (DMARDs) or other immunosuppressive drugs have been used to control disease activity. In more recent years, biological therapies or "biologics", have been added to the therapeutic arsenal, often showing very good efficacy and tolerability [26]. Other proposed novel therapeutic approaches include epitope-specific vaccination and the use of chimeric autoantibody receptor (CAAR) T cells [27, 28]. The success of biologics exemplifies and underlines the importance of detailed research on dysregulated immunological pathways to enable development of novel therapeutic approaches.

The precise nature of why autoimmune diseases occur is not known, yet it is clear that a combination of genetic and environmental factors is involved in their onset. Several studies have also determined significant gene-environment interactions, through which presence of one factor may influence the effect of another [29, 30].

1.2.1 Genetic factors in autoimmune disease

Genetic factors conferring increased susceptibility to autoimmune disease are evident through higher disease concordance rates between monozygotic twins compared to dizygotic twins or other siblings, with concordance rates of approximately 12-15% for monozygotic and 3-8% for dizygotic twins [31-33]. In the case of Sjögren's syndrome, a few case reports exist on twins concordant for the disease, but large twin studies are missing. Nevertheless, familial aggregation of the disease has been confirmed [34]. Given the shared immunopathological characteristics of several autoimmune diseases, such as those between Sjögren's syndrome and SLE, a familial clustering of various autoimmune diseases could be expected, and such observations are amply documented in the literature [35]. Consistently, variants in many genes or genetic loci have been associated with several autoimmune diseases.

1.2.1.1 Genetic variants associated with autoimmune disease

Monogenic variations or mutations that directly result in autoimmune diseases are very rare and often associate with severe disease phenotypes. Interestingly, by studying such monogenic diseases, pathways important for immune homeostasis have been pinpointed, as exemplified by studies of the IPEX syndrome and the *FOXP3* gene [18, 36]. Another example of a monogenic cause of autoimmunity is deficiency of the complement protein C1q, which is the strongest risk factor for SLE with a penetrance of nearly 100% in both sexes [37].

Genome-wide association studies (GWAS) have pinpointed common genetic variants which associate with increased or decreased risk of autoimmune diseases in a multitude of loci. However, these variants, usually single nucleotide polymorphisms (SNPs), only convey modest effects. Some genetic variants have, as mentioned above, been found to confer increased risk of several autoimmune diseases, thus implying shared immunopathological pathways [38]. A prime example is the *human leukocyte antigen* (*HLA)* region on chromosome 6, where the strongest genetic associations are found. However, extensive linkage disequilibrium among *HLA* alleles and vast genetic variability in the locus has hampered precise understanding of these associations.

Hundreds of non-HLA risk loci have also been identified, out of which many are important in immune responses such as T cell signaling and differentiation, immune cell activation, innate immunity, and TNF signaling [39]. Specifically, genes in which polymorphisms associate with multiple systemic autoimmune diseases include e.g., *STAT4, IRF5, PTPN22* and *FAM167A-BLK* [40, 41].

1.2.2 Environmental factors in autoimmune disease

The observation that concordance rates of autoimmune diseases in monozygotic twins are rather low [31-33, 42] and that heritability estimates are far from 100% [43] highlights the fact that genetics alone does not fully explain the development of autoimmune diseases. Consequently, environmental factors must play an important role. Indeed, for several autoimmune diseases, there is strong epidemiological evidence of associations between environmental factors and disease development, including exposure to smoking, silica, and solvents [44]. Moreover, reports of individuals developing autoimmune-like disease upon exposure to pharmaceutical drugs including IFN-α, antifungals, and angiotensin converting enzyme inhibitors [45-47] further support that exogenous agents can have a causative effect.

1.2.2.1 Identified and suggested environmental risk factors

In a comprehensive review, an expert panel assembled by the National Insitute of Environmental Health Sciences evaluated the evidence for the importance of various environmental factors in the development of autoimmune diseases [44]. The panel expressed confidence that silica exposure is a risk factor for development of several autoimmune diseases, that smoking is a risk factor for development of seropositive rheumatoid arthritis (RA), that solvent exposure is a risk factor for development of systemic sclerosis, and that ultraviolet radiation exposure is a protective factor for multiple sclerosis (MS). However, many other risk factors have been linked to development of autoimmune diseases, with varying degree of certainty. Specifically, smoking has also been associated with development of MS [48-50], SLE [51, 52], anti-Jo-1 antibodies in myositis [53], and, as mentioned above, seropositive RA [54]. Interestingly, gene-environment interactions have been determined between *HLA* alleles and smoking in RA [29] and MS [48], in which smokers carrying a risk genotype have much-increased odds ratios (ORs) compared to non-smokers without risk genes. Further, silica exposure has been reported to increase the risk of developing f RA [55], SLE [56], and systemic sclerosis [57]. Other noteworthy associations between environmental factors and disease development include low vitamin D and adolescent obesity with development of MS [58], sunlight exposure with flare of SLE [59, 60], and exposure to textile dust with development of RA [61].

Infections have long been suggested as risk factors for development of autoimmune diseases. For instance, a role of Epstein-Barr virus (EBV) infection has been suggested in MS [58], RA [62], SLE [63, 64], and Sjögren's syndrome [65]. Mechanisms by which viral infections may induce autoimmunity, and different viruses implicated in autoimmune diseases including Sjögren's syndrome have been reviewed by myself together with colleagues [66] and others [67] elsewhere.

Exposure to various environmental factors may be of different importance during certain timeframes in the autoimmune process and will interact with genetic risk factors carried by the individual, as depicted for Sjögren's syndrome below (**Figure 3**).

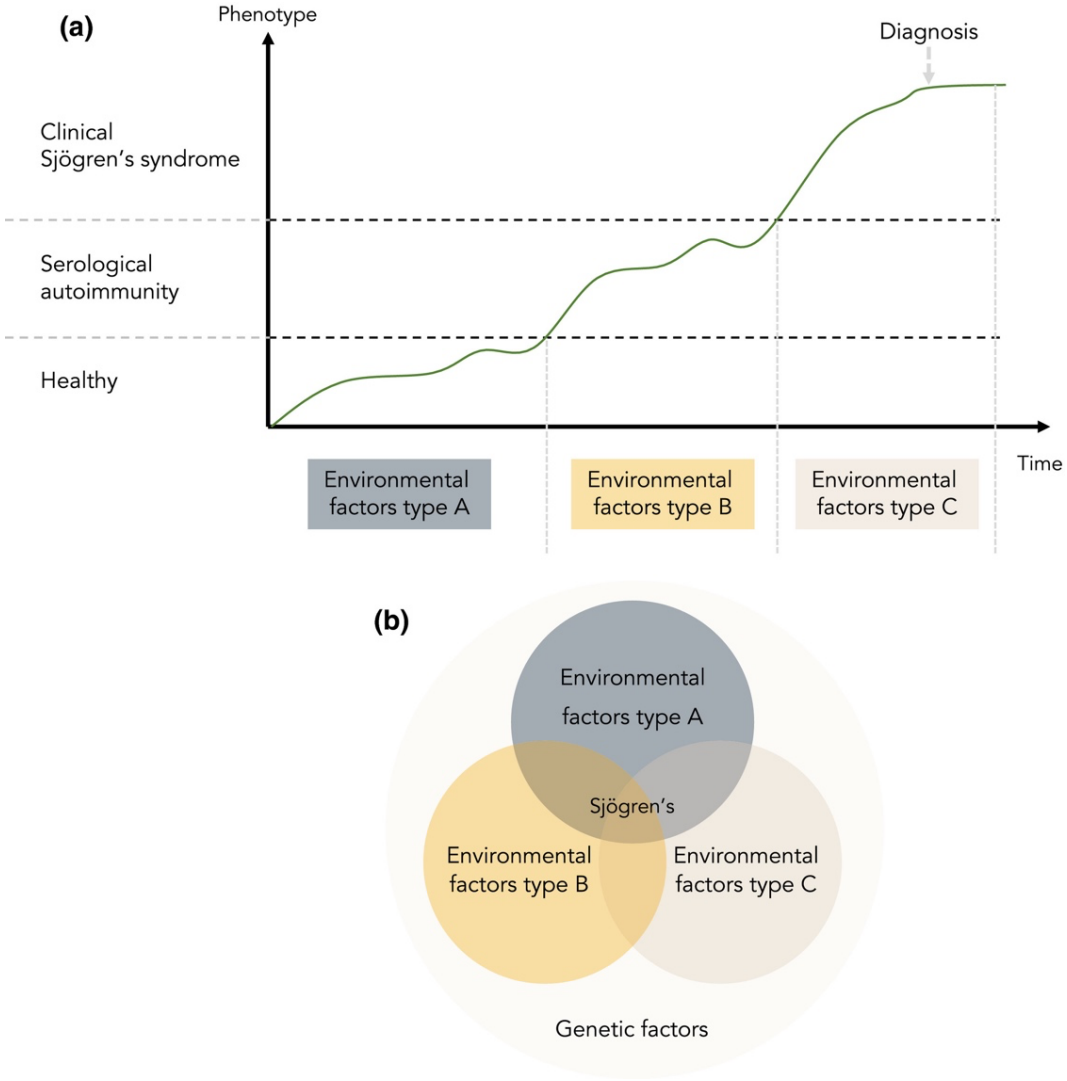

***Figure 3**. Suggested model of the contribution of environmental factors in the autoimmune process of autoantibody positive Sjögren's syndrome. (**a**) The disease progression of a genetically susceptible individual is indicated by the green line. Various environmental factors (A-C) contribute risk or protection during different stages of the disease process. Certain factors may be important e.g., for triggering serological autoimmunity while other, or the same, factors may influence disease severity. (**b**) Different environmental factors are likely to interact both with underlying genetics, as well as with each other. Reprinted with permission from [66].*

1.3 SJÖGREN'S SYNDROME

Primary Sjögren's syndrome is a systemic autoimmune disease characterized by hypofunction of exocrine glands, principally the salivary and lacrimal glands, resulting in the cardinal symptoms of dryness of eyes and mouth, which are commonly referred to as sicca symptoms. The disease is often termed primary when occurring isolated, and secondary when

occurring in the presence of another systemic autoimmune disease. This thesis is based on studies of the primary form of the disease and no patients with secondary Sjögren's syndrome have been included. Therefore, for the purpose of conciseness, the term Sjögren's syndrome is used in this thesis and refers to the primary form of the disease.

The prevalence of Sjögren's syndrome has been estimated at about 0.05-0.21% [68-70], and a substantial gender bias is observed with a female to male ratio of at least 10 to 1 [71, 72]. The incidence peaks around 40-50 years of age, however, there is usually a considerable delay between symptom onset and diagnosis. In addition to the oral and ocular dryness, patients may also experience symptoms such as fatigue, arthralgia, xeroderma, low-grade fever, and symptoms of exocrine dysfunction of mucosal surfaces in the oropharynx, bronchi, gastrointestinal tract, and in women, the vagina. Approximately 30 to 40% of patients develop a more systemic phenotype with organ-specific extra glandular manifestations (EGMs) that may involve e.g., arthritis, synovitis, cutaneous vasculitis, polyneuropathy, interstitial lung disease, and tubulointerstitial nephritis.

The morbidity associated with Sjögren's syndrome should not be underestimated, and overall mortality rates are increased [73]. Common symptoms such as fatigue, sicca symptoms, and arthralgia can have considerable impact at the individual level and may cause inability to work and decreased social interactions. In fact, patients with Sjögren's syndrome report a reduction in quality of life and health-related quality of life at levels comparable to patients with RA and SLE [74], and one study found even higher rates of psychological distress compared to patients with SLE [75].

1.3.1 Autoantibodies

Several types of autoantibodies can be found in the circulation of patients with Sjögren's syndrome and autoantibodies against Sjögren's-syndrome-related antigen A (Ro/SSA) and Sjögren's-syndrome-related antigen B (La/SSB) are detected in approximately 70% and 50% of patients, respectively [76]. Intriguingly, disease-associated autoantibodies have been observed in sera collected as early as two decades before diagnosis [77, 78], indicating that humoral autoimmunity may be initiated many years prior to clinically overt disease (**Figure 3**). Detection of anti-Ro/SSA and anti-La/SSB autoantibodies is associated with earlier disease onset, more severe disease with higher prevalence of EGMs, and correlate with various laboratory features such as leukopenia, thrombocytopenia, hypergammaglobulinemia, and higher expression of IFN stimulated genes (ISGs) [79-81]. Notably, stratification of patients by anti-Ro/SSA and/or anti-La/SSB positivity reveals genetic differences between autoantibody-positive and autoantibody-negative patients, and in fact, the *HLA* associations are exclusive to patients positive for these autoantibodies [82, 83].

1.3.2 Classification criteria

Classification criteria have been developed in order to standardize diagnosis for patients taking part in research studies, but these criteria are also widely used by physicians for establishing diagnosis in the clinical setting. Over the years, numerous sets of criteria have been proposed for classification and/or diagnosis of Sjögren's syndrome and between 1965 and 2002 no less than eleven such criteria were published [84]. However, until 2002 no

criteria had been endorsed by the American College of Rheumatology (ACR) or the European League Against Rheumatism (EULAR) [85]. The first set of classification criteria to be accepted by these organizations were the 2002 American-European Consensus Group (AECG) criteria [86], which are the criteria employed for classification of patients in the studies of this thesis. A new set of classification criteria were endorsed by the ACR in 2012 [87] but never reached wide usage, mainly since the older criteria performed well, robust validation was lacking, and also because of a lack of resources to perform the required ocular staining score. The latest classification criteria are the 2016 ACR/EULAR classification criteria [85], which are similar to, and have a high level of concordance with the AECG criteria [85, 88].

The 2002 AECG and the 2016 ACR/EULAR criteria are much alike in the sense that key elements of both comprise objective proof of ocular and salivary gland involvement, minor salivary gland biopsy, and presence of Sjögren's syndrome-associated autoantibodies. In both sets of criteria, signs of inflammation in minor salivary gland biopsies are a prerequisite for diagnosis for individuals where Sjögren's syndrome-associated autoantibodies are not detected in serum. In contrast to the 2002 AECG criteria, the 2016 ACR/EULAR criteria use a weighted scoring system. Moreover, the questionnaire for glandular symptoms included as part of the 2002 AEGC criteria has been changed to be used as inclusion criteria before applying the 2016 ACR/EULAR criteria. One important difference comparing the 2016 ACR/EULAR and the 2002 AECG criteria is that individuals who are anti-La/SSB positive but anti-Ro/SSA negative will no longer be classified as Sjögren's syndrome unless they have a positive biopsy. The decision to not include anti-La/SSB autoantibodies as part of the new criteria was based on results from statistical analyses showing that exclusion of this parameter did not affect classification performance [85]. Also contributing to the exclusion of anti-La/SSB were expert group discussions and a study by Baer et al. showing that serological presence of anti-La/SSB without anti-Ro/SSA is not significantly associated with Sjögren's syndrome phenotypic features [89].

1.3.3 Disease activity index

Monitoring of disease activity in clinical studies is commonly performed using the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [90] and the patient-reported scoring index EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) [91]. In the ESSDAI, scores from twelve organ-specific domains are weighted into a final index. A criticism of the index has been that the many patients who only present with sicca symptoms score very low on this index and therefore cannot be adequately monitored, while patients with systemic manifestations and several EGMs may score very high, since the index score ranges from 0 to 123. The ESSPRI score is used as a complement to the ESSDAI, and it is calculated as the mean of three subjective symptom scales graded 1-10: dryness, fatigue, and pain. Correlation between the two scores is poor [92, 93], indicating that the ESSDAI and ESSPRI scores are complementary and should be evaluated separately [93].

1.3.4 Genetic factors

Several candidate gene studies, as well as two large association studies, have identified associations between genetic variants and risk for development of Sjögren's syndrome [41, 94, 95]. One study in Caucasians revealed genome-wide significant associations with the *HLA* region as well as the *STAT4*, *IL12A*, *TNIP1*, *IRF5*, *BLK-FAM167A*, and *CXCR5* loci [94]. The associated genes are important in processes such as antigen presentation, lymphocyte signaling, the NF-κB pathway, and the IFN signaling pathway [41].

1.3.5 Environmental factors

Environmental risk factors for development of Sjögren's syndrome have been little investigated. Although a link to various viral infections has been repeatedly suggested, convincing data support this hypothesis are lacking. Myself together with colleagues have previously reviewed environmental factors that may be of importance in the pathology of Sjögren's syndrome [66]. In summary, studies on exposures prior to diagnosis have indicated possible associations between Sjögren's syndrome and exposure to infections in general, nontuberculous mycobacterial infection, helicobacter pylori infection, low lifetime exposure to estrogen, deficiency of vitamin D, stress, former smoking, and silicone breast implants [66]. Importantly, however, although several studies have indicated association with infections in general, all potential risk factors need further study and thus far no single environmental risk factor for Sjögren's syndrome has been unambiguously identified.

1.3.6 The immunopathology of Sjögren's syndrome

Sjögren's syndrome shares several common features with other systemic autoimmune diseases such as production of autoantibodies, formation of ectopic lymphoid structures, high levels of several pro-inflammatory cytokines, and activation of the IFN system. Thus, the immunopathogenesis involves both innate and adaptive immunity. Specifically, aberrances in B cell function and persistent production of type I IFN have emerged as two central features.

1.3.6.1 Immune cell aberrations

Several aspects of the disease imply chronic activation of B cells, a feature that remains incompletely understood. Patients display disturbed peripheral B cell subpopulation frequencies with more naïve B cells and fewer $CD27^+$ memory B cells compared to controls [96, 97]. Consequences of the B cell overactivity include hypergammaglobulinemia and production of autoantibodies toward the ribonucleoproteins SSA (Ro52 and Ro60) and SSB (La). Interestingly, autoantibody production partly occurs locally in inflamed salivary glands [98], where the Ro52 autoantigen has been shown to be present [99] and higher local autoantibody production is observed in patient salivary glands containing GC-like structures [100]. Such structures are observed in salivary glands from 30-40% of patients [101], and their presence is associated with an increased risk of development of B cell lymphomas [102, 103]. Of note, genetic variation close to the gene locus of the chemokine receptor *CXCR5* locus has been linked to Sjögren's syndrome [94], and intriguingly, CXCR5 and its ligand CXCL13 are central in the formation and organization of GC-like structures [104, 105].

Overall, several lines of evidence point to the importance of B cells and GC-like structures in the pathology of Sjögren's syndrome.

T cells, predominantly $CD4^+$, are the major infiltrating cell type the salivary glands at early stages of the disease [106], and strong genetic association between Sjögren's syndrome and the *HLA* locus [94] further highlights their importance. T cell polarization toward Th1 and Th17 phenotypes is believed to contribute to the inflammation and disease [107, 108].

Salivary gland epithelial cells (SGEC) may actively contribute to propagation of the disease through expression of co-stimulatory molecules such as CD86 [109] and release of inflammatory molecules such as B cell activating factor (BAFF) and CXCL13 [108, 110]. Moreover, increased apoptosis of SGECs could be an important cause of sustained autoantigen exposure [111].

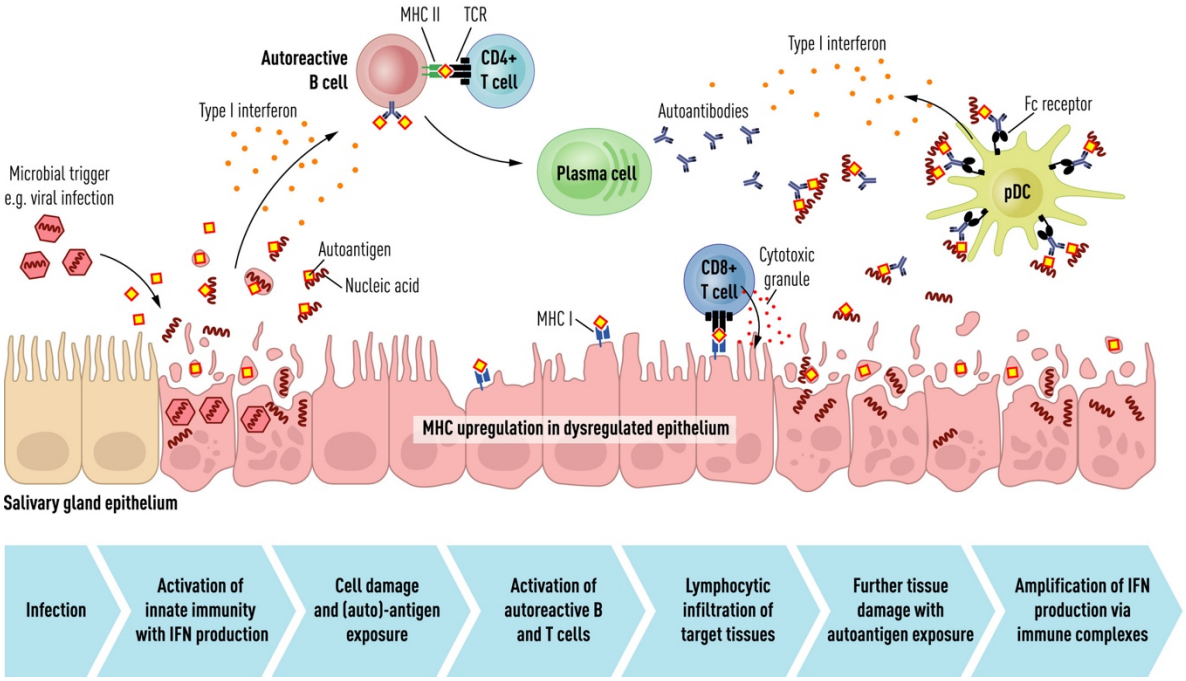

***Figure 4**. Schematic illustration of proposed mechanisms underlying Sjögren's syndrome. Infections disrupt the salivary gland epithelium causing an inflammatory microenvironment with production of type I IFN and release of autoantigens and microbial antigens from dying cells. Subsequently, microbial and self-antigens are processed and presented, whereby autoreactive B and T cells are activated, in turn causing B cell differentiation into autoantibody producing plasma cells. The autoantibodies form immune complexes with autoantigens, which are endocytosed by pDCs, causing further production of type I IFNs. In turn, the type I IFNs promote differentiation and activation of autoreactive B cells thus causing increased autoantibody production. Hereby, a self-sustaining cycle of autoimmunity is created. (pDCs, plasmacytoid dendritic cells; TCR, T cell receptor; IFN, interferon; MHC, major histocompatibility complex). Reprinted with permission from [66].*

1.3.6.2 Disturbed signaling pathways

A wide set of inflammatory cytokines and chemokines are expressed at high levels in Sjögren's syndrome, including type I IFNs which are often thought of as key pathogenic mediators (**Figure 4**). After initial reports of increased type I IFN activity and accumulation of IFN-producing plasmacytoid dendritic cells (pDC) in salivary glands of patients [112,

113], augmented activity of the type I IFN system was also reported systemically in peripheral blood cells [114]. A so called “IFN signature” is present in about 55% of patients and presence of the signature correlates with disease activity, higher titers of anti-Ro/SSA and/or anti-La/SSB autoantibodies, and hypergammaglobulinemia [115]. Therefore, patient stratification based on the presence of the signature has been suggested. The fact that genetic variants in the *STAT4* and *IRF5* loci have been associated with Sjögren’s syndrome further implies the importance of type I IFN pathways in the disease [94, 116]. Because of the anti-microbial properties of type I IFNs, it has been suspected that infections may act as initiators of their uncontrolled signaling (**Figure 4**).

Plasmacytoid dendritic cells have been identified as main producers of type I IFN and are often considered to be of central importance in the pathogenesis of Sjögren’s syndrome. It has been proposed that presence of autoantibodies results in RNA-containing immune complexes which stimulate IFN production by pDCs [112]. The secreted type I IFNs in turn stimulate other cells of the immune system and lead to increased inflammatory cytokine production. Specifically, type I IFN can induce the production of BAFF via IRF1 and IRF2 [117], and BAFF in turn promotes autoantibody production in B cells [118, 119]. Hereby, a vicious and self-propagating cycle of immune activation is established (**Figure 4**) [120].

The importance of BAFF in the pathology of Sjögren’s syndrome is well established [121] and levels of BAFF in serum have been shown to correlate with ESSDAI scores [122]. Interestingly, transgenic mice overexpressing BAFF develop Sjögren’s syndrome-like pathology with age [123], and overexpression of BAFF in a Sjögren’s syndrome mouse model was shown to result in enhanced lymphocytic infiltration, and B cell activation and differentiation in the target tissue [124].

1.3.7 Treatment

To this day, no immunomodulatory treatments have been proven efficacious for treatment of Sjögren’s syndrome. While muscarinic agonists have been shown to be beneficial for treating oral and, to some extent, ocular dryness [125], these drugs are associated with side effect which limit their use. Therefore, treatment is focused on relieving symptoms related to dryness by using topical agents such as moisture replacements for eyes and mouth, and local estrogen treatment to relieve dryness of the vagina.

Hydroxychloroquine (HCQ) is an antimalarial drug used in the treatment of several rheumatic diseases. In SLE, HCQ has been shown to reduce the number and severity of clinical flares, and to improve overall survival [126]. Although HCQ is sometimes used for treatment of Sjögren’s syndrome, the only randomized clinical trial conducted to date could not determine efficacy of the drug after 24 weeks of treatment [127]. However, a small randomized controlled clinical trial of leflunomide-HCQ combination therapy indicated significant reduction of ESSDAI scores at 24 weeks, but this recent trial was small and this combination of the drugs needs to be studied further [128].

Several trials of biological drugs have been conducted. Randomized controlled trials of anti-TNF treatment have not been successful [129, 130], likewise, a recent trial of abatacept (blocking of CD28-CD80/CD86 co-stimulation) failed to reach the primary outcome [131].

However, a recent proof-of-concept study of iscalimab (anti-CD40 blocking the CD40/CD40L pathway) showed preliminary efficacy, with significant reduction in ESSDAI score after 12 weeks of treatment compared to placebo [132], thus warranting further studies.

In line with the central role of B cells in the pathogenesis of Sjögren's syndrome, therapies aimed at B cell abrogation have been assessed. Although treatment with rituximab (anti-CD20 mediated B cell depletion) has been shown to decrease the number of B cells, GCs, and lymphoepithelial lesions in parotid gland biopsies [133], randomized clinical trials failed to reach their primary endpoints [134, 135]. Further, an open-label phase II study investigated the effect of belimumab (BAFF inhibition), in which 60% of the treated patients reached the primary endpoint, indicating a possible efficacy [136]. Recently, potential efficacy of ianalumab (B cell-depleting, BAFF receptor-blocking) was indicated in a phase IIb study [137].

Difficulties in finding efficacious drugs for treatment of Sjögren's syndrome partly relate to heterogeneity of the disease, and to challenges in finding accurate measurements of disease activity.

1.3.8 Comorbidities and complications

An important complication associated with Sjögren's syndrome is development of lymphoma, most commonly low-grade non-Hodgkin lymphoma, with a lifetime risk of approximately 5% [103, 138]. Predictors of lymphoma development include lymphopenia, low complement protein levels, cryoglobulinemia, monoclonal component in serum or urine, permanent swelling of salivary glands, splenomegaly and lymphadenopathy, and palpable purpura [139].

Patients are also at increased risk of developing cardiovascular disease, with an especially pronounced risk in those positive for anti-Ro/SSA and anti-La/SSB autoantibodies [140]. Moreover, presence of the anti-Ro/La autoantibodies in pregnant women can result in development of neonatal lupus syndrome in the child, which may include the rare complication congenital heart block [141].

1.4 SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus is a heterogeneous disease characterized by multi-organ involvement and production of a vast set autoantibodies. In the 1950s, the 5-year survival rate was estimated to be less than 50% [142], but this figure has dramatically increased to more than 95% [143], partly owing to earlier diagnosis, better understanding of the disease, and new therapeutic regimens. The disease predominantly affects women of fertile age but can occur at any age in both sexes. There is a considerable variance in the reported prevalence and incidence across the world [144]. In Sweden, the estimated prevalence of SLE is 65/100 000 with an annual incidence of about 2.8-5/100 000 [145, 146].

The diagnosis can be established in the presence of disease manifestations from more than one organ system and at least one characteristic autoantibody. Classification criteria originally developed for research studies are often used to support the diagnosis, and the most

commonly used are the 1982 ACR criteria where at least 4 out of 11 criteria need to be fulfilled for classification as SLE [147]. Disease activity in clinical studies is typically monitored using the SLE Disease Activity Index (SLEDAI) [148].

Large numbers of genetic association studies have revealed more than 100 loci associated with SLE, but each locus usually with a small effect on risk [149]. Interestingly, some genetic variations have been linked to specific organ manifestations or comorbidities. Variation in *STAT4* has been associated with production of dsDNA-autoantibodies, severe renal insufficiency, and stroke [150-152]. Likewise, genetic variation in *IRF8* has been associated with coronary heart disease [153]. Suggested or confirmed environmental risk factors include ultraviolet radiation, smoking, exposure to silica, and infections [44].

The immunopathology of SLE shares many features with that of Sjögren's syndrome, including augmented activity of B cells and the type I IFN system. However, SLE is also characterized by high amounts of exposed autoantigens, which is reflected by the fact that more than 100 autoantibodies have been described [154]. Two major causes of high abundance of autoantigens in SLE is the inability to clear debris from dead cells, and impaired degradation of neutrophil extracellular traps [155, 156]. Improperly cleared immune complexes containing nucleic acids are internalized by pDCs via FcγRIIa, whereupon the nucleic acids interact with TLR7 and TLR9 in endosomes and stimulate production of type I IFN [157, 158]. The central importance of type I IFN in the pathogenesis of SLE is supported by observations of development of SLE, or symptoms associated with SLE, in individuals treated with IFN-α [45, 159].

Treatment of SLE depends on the type of organ involvement and severity of the flare. The antimalarial drug HCQ is known to prevent flares and prolong life and is therefore recommended to all patients if there are no contraindications. Antimalarial agents are otherwise used especially for skin and joint involvement. Systemic corticoids are effective in relieving symptoms and decreasing inflammation. Immunosuppressive drugs such as methotrexate, azathioprine, cyclosporine, and mycophenolate mofetil are also used. Regarding biological treatment, several drugs are used off-label. Thus far, the BAFF-inhibiting drug belimumab is the only officially approved biological agent for treatment of SLE. However, anifrolumab, a monoclonal antibody targeting IFNAR1 was recently shown to be efficacious in patients with SLE [160], which will be discussed later in this thesis.

1.5 VACCINATION IN RHEUMATIC DISEASES

Prevention of infectious diseases through vaccination undoubtedly represents one of the greatest accomplishments throughout the history of medicine. Importantly, patients with rheumatic diseases are at increased risk of infection [161, 162] and a study by Brito-Zerón et al. identified infections as the second most common cause of death, after cardiovascular disease, in patients with Sjögren's syndrome [73]. Consequently, there is an undisputable need to protect patients with rheumatic diseases against infections.

Large, randomized studies assessing efficacy and safety of vaccinations in patients with rheumatic diseases are lacking. Also, vaccine efficacy is often estimated by measuring

serological responses i.e., titers of neutralizing antibodies, which do not always correspond well to clinical protection. Because of variations in study design, type of included patients, differences in treatments, and differences in vaccines used, many studies show conflicting results regarding vaccine efficacy, and thus, firm conclusions are generally difficult to make. The efficacy of various vaccines in patients with different rheumatic diseases and different treatments has been extensively reviewed elsewhere [163-165]. Briefly, most vaccines seem to be effective in patients with rheumatic diseases, but for some vaccines and for some treatment groups the response to vaccination is lower in patients compared to controls. Vaccination against H1N1 influenza in patients with SLE, RA, ankylosing spondylitis, psoriatic arthritis, and dermatomyositis is one such example, in which these patient groups have significantly reduced seroprotection rates compared to healthy controls [166-168]. B cell-depleting treatment using rituximab is the therapy most consistently associated with poor responses to vaccinations [163, 164], and consequently, the 2019 EULAR recommendations state that vaccines ideally should be administered before start of such therapy [169].

2 AIMS OF THE THESIS

Autoimmune diseases are thought to occur through combinations of genetic and environmental risk factors. Although primary Sjögren's syndrome is one of the most prevalent rheumatic diseases, much remains unknown about its etiology and immunopathogenesis. The aims for the work performed in this thesis were to identify specific environmental factors of importance for development of Sjögren's syndrome and to improve understanding of dysregulated immune processes of adaptive and innate immunity, with a focus on *in vivo* immune responses to microbial antigens.

The specific aims of the thesis were:

- To perform epidemiological studies using questionnaires and the Swedish health care registers for identification of environmental factors of importance for development of Sjögren's syndrome

- To identify and describe differences in immune activation *in vivo* after microbial antigen exposure comparing patients with systemic autoimmune disease and controls

- To establish novel methods for quantification of IFN system activation, which do not rely on RNA samples

- To define cellular immune dysregulation in Sjögren's syndrome by obtaining an in-depth understanding of differential B cell gene expression patterns and by exploring the CXCR5-CXCL13 axis in peripheral blood and salivary glands

3 METHODOLOGICAL CONSIDERATIONS

In the following section, select methods included in this thesis are discussed with a focus on limitations and advantages. Detailed descriptions of each method are available in the individual papers.

3.1 STUDY POPULATIONS

All papers included in this thesis include data or biological samples collected from patients with Sjögren's syndrome or SLE, and from controls. To ensure correctly classified patients and avoid errors which, for instance, may occur when selecting patients based on ICD codes, we chose to include only patients with Sjögren's syndrome fulfilling the 2002 AECG criteria [86] and patients with SLE fulfilling the revised 1982 ACR criteria for SLE [147], which had been evaluated by a rheumatologist seeing the patient. However, new classification criteria for Sjögren's syndrome were published during the work leading up to this thesis [85]. While the new criteria do not consider isolated anti-La/SSB autoantibodies as a marker of Sjögren's syndrome, they classify individuals in a similar manner as the previous AECG criteria [88] and we therefore have no reason to believe that implementation of the new criteria would have a significant impact on results presented in this thesis. Nevertheless, in **Paper I and II**, we used the presence of anti-La/SSB autoantibodies for stratification of the cohorts. Although the presence of anti-La/SSB autoantibodies alone does not seem to be a valuable marker for classifying patients [89], we would like to underline the value of considering the presence of these autoantibodies for patient stratification, which will be discussed later in the thesis.

The clinical and immunological heterogeneity of Sjögren's syndrome has posed great challenges both when dissecting the immunopathology of the disease, and in clinical trials aimed at identifying effective therapies. In efforts to reduce variation introduced by such heterogeneity, we in **Paper III and V-VII** chose to mainly study patients positive for anti-Ro/SSA autoantibodies. Whereas this may reduce inter-individual variability between patients, a consequence is that the observations may not be applicable to all patients with Sjögren's syndrome. However, with the emergence of more personalized medicine, we believe that studies of selected patient subgroups are of great importance.

3.2 GENE EXPRESSION ANALYSIS

Nanostring technology was applied for gene expression analysis in **Papers III, V, and VI**. This method utilizes fluorescently labelled probes for direct quantification of mRNA molecules, thus eliminating the need of generating cDNA by reverse transcription and subsequent amplification steps. The process is largely automated on the nCounter system, thus further reducing variability that may otherwise be induced during sample processing. However, Nanostring gene expression analysis is limited to exploration of pre-defined genes lists, in our case the Human Immunology CodeSet v2 entailing 594 genes. We chose this method partly due to its lower cost and availability in our research environment, but also because of its need for very small amounts of RNA input (50 ng total RNA). We had opted

for studying sorted cell populations and were consequently limited by amounts of purified total RNA, mainly due to low cell counts in some patient samples. It would have been advantageous with a less biased approach to gene expression analysis in the form of either microarrays or RNA sequencing. Indeed, in **Paper VI** we employed whole transcriptomic analysis by RNA sequencing and were able to detect >4000 differentially expressed genes in $CD19^+$ B cells comparing patients and controls.

3.3 PROTEIN MEASUREMENTS

To measure protein levels in serum and plasma, we used Olink proximity extension assay (PEA) technology which is a multiplexed assay determining levels of pre-selected protein panels [170]. The PEA method utilizes dual antibody recognition of each target meaning that two antibodies coupled to DNA oligonucleotides need to bind epitopes in close proximity, and thereafter hybridize, for a target to be detected. Advantages of this method are therefore the high specificity in detection of targets, the possibility of creating multiplexed assays with high throughput, as well as the small sample volume needed (one µl of serum or plasma). We selected the Immuno-Oncology panel encompassing 92 proteins for our studies since it contained the largest number of proteins in our interest. Although many proteins were found to be differentially abundant in the circulation comparing patients and controls, the understanding of proteomic differences is limited due to the pre-selection of proteins. For this reason, we did not perform any pathway enrichment analyses on the protein data, since the limited number of proteins that are already selected for their relevance in immunology would skew such an analysis toward detection of immunological pathways.

When designing the studies resulting in **Paper III and IV**, we selected time points optimal for measuring serological responses (day 0, 28 and 90) and induced gene expression (day 0 and day 1) following vaccination. However, we also aimed to determine perturbations in the plasma proteome. Although we could detect significant differential changes for three proteins comparing the control and patient group (**Paper III**, Figure 2B), we realized that twenty-four hours may be a too short interval for optimal studies of changes in the circulating proteome. In light of this, we did not perform a corresponding proteome analysis in **Paper IV** encompassing both day 0 and day 1, but instead focused our efforts on understanding the proteomic profile prior to vaccination and how it associated with serological responses.

3.4 QUANTIFICATION OF INTERFERON SYSTEM ACTIVITY

The large number of different type I IFN molecules together with their low concentrations during normal physiological conditions make their direct measurements challenging [1]. For this reason, expression of ISGs is often used as a proxy for quantification of interferon system activation. The expression of selected panels of such genes can be normalized against a control group and summed up into a so called “IFN score”, calculated as a sum of Z-scores. In our studies, we have used a previously published formula [171] for generation of several different types of IFN scores:

$$IFN\ score = \sum_{i=1}^{n} \frac{Gene\ i_{patient} - mean\ Gene\ i_{controls}}{SD(Gene\ i_{controls})}$$

Although methods for direct quantification of type I IFNs have been developed, such as the SIMOA assay for IFN-α [172], no such assay suitable for routine clinical assessment of IFN system activity is available. In **Paper V**, two novel methods for measuring IFN system activity are presented in the protein IFN (pIFN) score and the DNA methylation (DNAm) IFN score. However, the pIFN score is based on PEA technology and the DNAm IFN score on DNA methylation analysis on an Illumina HM450K BeadChip. Both these methods are costly and difficult to implement in clinical practice, wherefore we suggest these scores to be used primarily in situations where gene expression analysis for calculation of an IFN score is not possible.

4 RESULTS AND DISCUSSION

In this section, the papers included in this thesis will not be discussed in the order indicated by their Roman numerals. Instead, to achieve better logic, the papers will be discussed in a manner starting with exogenous factors, thereafter endogenous factors, and lastly perturbed endogenous factors.

4.1 ENVIRONMENTAL FACTORS IN SJÖGREN'S SYNDROME

At the initiation of the work leading up to this thesis, few studies had investigated potential environmental risk factors for Sjögren's syndrome. Moreover, most of the available studies had been performed in prevalent cases and analyzed current exposure, not specifically considering exposures occurring before diagnosis. Logically, risk factors will exert their effect before or during initial stages of the disease development, preceding established diagnosis. For a disease like Sjögren's syndrome, which typically has an insidious onset spanning several years, this presents considerable challenges. Partly owing to non-specific symptoms such as fatigue, dryness, and arthralgia, the diagnosis may be delayed both due to diagnostic challenges for the physician as well as the patient's hesitance to seek medical care. Indeed, in the cohort studied in **Paper II**, the patient-reported average delay between symptom onset and diagnosis was 6.2 years. A further challenge when assessing possible environmental risk factors for Sjögren's syndrome is that dryness of mucosal tissues may act to change behavior due to discomfort, and that the disease itself may impair innate mucosal barrier functions. In addition, it is plausible that different environmental factors could be important for diverse and heterogenous sub-phenotypes of the disease with separate symptomatic, biologic, and genetic characteristics. In all, risk factor studies in Sjögren's syndrome pose particular challenges which must be taken into special consideration.

Identification of environmental risk factors depends on high-quality exposure data and the commonly used retrospective case-control studies have an inherent risk for recall bias and misclassification errors. Furthermore, it is important to keep in mind that whereas observational studies are critical for identifying potential environmental risk factors, they cannot prove causation. Confirmation of results from observational studies in multiple populations strengthens possible causation, but only experimental studies determining how agents break immunological tolerance can provide final proof. Interactions between specific genotypes and risk factors [173, 174], variability in disease phenotypes, critical time frames of exposure, and hormonal changes during the lifespan are factors further adding to the complexity of epidemiological risk factor studies.

In our efforts to assess potential environmental risk factors for Sjögren's syndrome, we chose to focus on cigarette smoking (**Paper I**) since smoking is an established risk factor for several autoimmune diseases such as RA [54] and MS [48], and infections (**Paper II**) since microbial triggers have long been suspected to be important in the pathogenesis [107].

4.1.1 Detailing smoking patterns preceding diagnosis

To assess the influence of smoking on the development of Sjögren's syndrome, we performed a questionnaire-based case-control study in our national Swedish patient cohort and matched controls from two previous studies in RA [54, 175]. A total of n=815 patients were invited to participate in the study, out of which n=606 patients (74%) responded to the questionnaire, making our study the largest questionnaire-based risk factor study performed in Sjögren's syndrome. During subsequent matching of up to n=15 controls per patient, some patients could not be successfully matched and thus the final number of patients included in the analyses was n=530. Reassuringly, demographics of these patients were similar to those of the total invited cohort (**Paper I**, Table 1). The index year was defined as the year of diagnosis for patients, and the year of responding to the questionnaire for controls. Odds ratios (ORs) were estimated by logistic regression conditioned on the matching by age at index date, calendar time at index date, sex, and area of residency.

4.1.1.1 Lower frequencies of smoking among cases

We observed lower rates of ever smoking (37% vs 44%) and current smoking (7% vs 16%) comparing patients and controls, which resulted in ORs of 0.67 (95% CI 0.55 – 0.81) and 0.37 (95% CI 0.26 – 0.53), respectively.

The observation of a lower frequency of current smoking is consistent with previous studies in prevalent cases [176-178], and could intuitively be explained by a lower likelihood of patients to smoke due to ocular, oral, and respiratory irritation caused by tobacco smoke. Another, but perhaps less likely, explanation for this observation would be that smoking decreases the likelihood of disease development. Cigarette smoke has indeed been described to have extensive immunomodulatory properties [179, 180]. Notably, one study employing the 2002 AECG criteria found lower frequency of focal sialadenitis and lower mean focus scores in minor salivary gland biopsies from currently smoking compared to non-smoking patients [177], and one older study using the previous Copenhagen classification criteria [181] showed similar results with significantly lower rates of abnormal focus scores in currently smoking compared to never smoking patients [182]. Such observations could however also be explained by a higher likelihood for patients with lower focus scores and less inflammation to continue smoking, possibly owing to milder disease symptoms.

Only one previous study has considered exposure to smoking prior to diagnosis and in that study, individuals who would later develop Sjögren's syndrome were less probable to be current smokers, but more likely to be former smokers, thus suggesting former smoking as a risk factor for disease [58]. In our study, estimates of former smoking were not significant and displayed consistent trends of ORs below 1, wherefore we have no indications that smoking is a risk factors for development of Sjögren's syndrome. This inconsistency could relate to differences in study design and the fact that in the study by Olsson et al., the exposure data had been collected in a health survey with a median time of 8.2 years prior to diagnosis.

Stratification of patients according to anti-Ro/SSA and La/SSB serology resulted in similar OR estimates for ever and current smoking in the autoantibody positive and negative

patient groups (**Paper I**, Table 2). I found this result surprising considering the genetic [82] and clinical [83] differences between these patient subgroups. However, this similarity could be interpreted as support for a hypothesis that early, and perhaps unrecognized, disease symptoms of dryness decrease the probability of smoking among the patient group as a whole.

4.1.1.2 Exposure to smoking over time

To better comprehend exposure to smoking over time, we estimated period prevalence of smoking in 10-year strata and depicted the smoking patterns in graphs (**Paper I**, Figure 1). The graphical visualization of smoking patterns preceding diagnosis led to the major finding of our study, namely, that smoking patterns were similar comparing cases and controls up until approximately 35 years before diagnosis, and that smoking thereafter declined in cases compared to the controls. To quantify these observations, we calculated likelihoods to stop smoking, which statistically supported a decline in exposure in patients relative to controls. Likewise, in these analyses, no large differences could be observed based on stratification by autoantibody status among the cases.

To my knowledge, our study is the first to describe smoking exposure patterns that precede diagnosis of Sjögren's syndrome at this level of detail. The interpretation of these results is however challenging and should be approached with care. Why does the prevalence of smoking decline already 30-40 years prior to diagnosis? Is the explanation that those who continue smoking are better protected against development of Sjögren's syndrome? Or is it rather so, that individuals who develop disease have early and unrecognized symptoms causing them to stop smoking already many years before diagnosis? Our study cannot provide definitive answers to these questions. On one hand, it is possible that cigarette smoke could act to diminish inflammation and that smoking discontinuation thus could trigger disease. For example, it is widely acknowledged that current smoking confers protection against ulcerative colitis (UC) [183]. In fact, heavy smokers present with milder disease compared to light smokers [184], and smoking patients are less likely to require corticosteroid treatment compared to non-smokers [185]. Intriguingly, UC, like Sjögren's syndrome is a disease affecting mucosal tissues, which could suggest shared influence of smoking on similar pathological processes between the two diseases. On the other hand, smoking has been identified as a risk factor for the immunologically related diseases SLE and RA [54, 186], wherefore it is difficult to mechanistically explain how the autoimmune processes would drastically differ in Sjögren's syndrome. Further explanations for our observations could be that some smokers attribute symptoms of dryness to their smoking, but may in fact have Sjögren's syndrome, and therefore remain undiagnosed. Alternatively, that physicians are less prone to investigate and diagnose symptoms of dryness in smoking individuals.

4.1.1.3 Smoking and risk-associated HLA

We, for the first time, had the possibility to assess potential interactions between risk-associated HLA haplotypes and smoking. This is of special relevance since smoking has been shown to interact with risk-associated HLA in seropositive RA [187] and MS [48]. We

selected HLA-DRB1*03 and HLA-DRB1*15 for our analyses, since these alleles have been associated with Sjögren's syndrome and production of autoantibodies [82, 94]. We also included HLA-DRB1*01/04/10 to mark shared epitope based on its interaction with smoking in RA [187]. Analysis of HLA haplotype frequencies in our cohort confirmed previous observations of the associations between HLA-DRB1*03 and Sjögren's syndrome (**Paper I**, Supplementary table 3). However, our analysis did not reveal any significant interactions between smoking and the investigated HLAs, which could perhaps be expected since smoking had not been identified as a risk factor for Sjögren's syndrome.

4.1.1.4 Complexities of the role of smoking in Sjögren's syndrome

A methodological limitation of our study is that the cases answered the questionnaire at a median time of 10 years after diagnosis, which could cause differences in recall bias between cases and controls. It would have been ideal to perform the study on incident cases, but we did not have that possibility at the time. Furthermore, recall bias may increase the longer back in time that exposure is analyzed. Nevertheless, this error would presumably not differ between cases and controls. We defined the year of diagnosis as the index date, however, it would be interesting and perhaps preferential to define the year of symptom onset as the index date. Such data were available for a considerable fraction of the cases in our study but changing the index date to the year of self-reported symptom onset did not have a large impact on the observations, wherefore those data were not included in the final manuscript.

Strengths of our study are the relatively high number of patients in comparison with previous studies, and that all cases were classified according to the AECG criteria [86]. Furthermore, we were able to stratify patients not only based on autoantibodies, but also based on risk-associated HLA, which had not previously been done.

In all, we show that smoking does not seem to increase the risk of developing Sjögren's syndrome and that patients compared to controls are more likely to stop smoking already several decades before diagnosis. The data may be interpreted in various ways and we cannot exclude the possibility that smoking confers protection. However, the observations could also be explained by behavioral changes due to symptoms of dryness appearing long before diagnosis. Future studies should be performed on incident cases, with detailed exposure data and careful patient stratification based on clinical symptoms, disease manifestations, serology, as well as genetics.

4.1.2 Infections as a conceivable trigger

As reviewed in the introduction of this thesis, viral infections constitute the main environmental factor discussed in the context of Sjögren's syndrome. The hypothesis of viral triggers partly stems from observations of increased activity of the anti-viral type I IFN system in a majority of patients [115], and from the involvement of TLR receptors recognizing endosomal nucleic acids [112]. Although previous studies have indicated higher frequencies of antibodies targeting viral proteins from e.g. EBV [188, 189] and human T-lymphocyte virus type 1 (HTLV-1) [190], these studies are all performed in prevalent cases and therefore do not indicate causation. To better comprehend possible connections between

previous infections and development of Sjögren's syndrome, we therefore utilized the unique possibilities of the Swedish national health care registers.

In **Paper II**, we used a well-characterized national cohort consisting of n=945 patients fulfilling the 2002 AEGC criteria [86]. Each case was matched by sex, age, and region of residency through the Swedish Total Population Register to n=10 control individuals from the general population (n=9048). Data on infections were then linked to each individual through the National Patient Registry, which includes data on hospitalizations since 1987 and outpatient care since 2001 but not patient visits to general practitioners. In an effort to reduce reverse causation, infections occurring within one year before Sjögren's syndrome diagnosis were not considered in the analyses. Odds ratios were estimated by conditional logistic regression.

4.1.2.1 Infections and disease development

Exposure to infections was higher in cases compared to controls (21.4% vs 12.9%), resulting in an OR of 1.9 (95% CI 1.6-2.3). Similarly to **Paper I**, we performed analyses stratifying cases based on autoantibody positivity and found that the association between a history of infections and anti-Ro/SSA and anti-Ro/SSB positive disease was stronger (OR 2.7, 95% CI 2.0-3.5) compared that of autoantibody negative disease (OR 1.9, 95% CI 1.4-2.7). Although the latter estimate supports a relationship between infection also with autoantibody negative disease, it is interesting that the association with anti-Ro/SSA and La/SSB autoantibody positive disease is stronger, which would indicate a mechanism whereby autoantibody production can be triggered by infections. This model fits well with observations made in **Paper III** where we could observe that titers of anti-Ro52 autoantibodies increased in untreated patients following viral antigen exposure, an observation that was also made in a previous study following adjuvanted anti-H1N1 influenza vaccination [191]. Indeed, transient autoantibody production has been described even in healthy persons following infection with e.g. hepatitis B and C, parvovirus B19, EBV, and human immunodeficiency virus [192]. Notably, patients with autoantibodies against Ro/SSA and La/SSB are more frequently IFN signature positive [115], younger at diagnosis (**Paper II**, Table 1), and more often carry risk-associated HLA (**Paper I**, Supplementary figure 3), which indicates that discrete autoimmune processes are at play in these individuals.

4.1.2.2 Routes of infection

To investigate various routes of infection, we specifically assessed infections in the gastrointestinal, respiratory, cutaneous, and urogenital systems since these sites represent main barriers for pathogen entry. We found that infections of the skin, respiratory tract, and urogenital system associated with development of Sjögren's syndrome. Interestingly, observations at these sites also displayed higher odds ratios in the autoantibody positive patient group, while only respiratory infections were found to associate with autoantibody negative disease (**Paper II**, Table 2). Gastrointestinal infections were not found to associate with disease. Considering the results in **Paper I**, it would be interesting to assess the association between respiratory infections and disease in the context of smoking, since smoking will predispose for such infections. However, data on smoking were unfortunately

not available at the time of the study. Indeed, smokers contract more bacterial and viral respiratory infections, including more and worse colds as well as influenzas, and also have increased risk of bacterial pneumonia [193]. Keeping in mind that smoking exposure was lower in patients compared to controls in **Paper I**, other causes of the higher rate of respiratory infections in patients need to be considered, including genetic and mucosal factors associated with Sjögren's syndrome. Undoubtedly, the mucosal dryness associated with the disease could be an important factor for increased susceptibility for infections and could constitute a cause of reverse causality in our study. However, even when excluding infections occurring during seven years prior to diagnosis in the analysis, the associations with respiratory infections still persisted (**Paper II**, Figure 2). In future studies, we therefore aim to study infections as a risk factor in the context of both smoking and genetic factors.

4.1.2.3 Infections as triggers of Sjögren's syndrome

Our observations of an association between infections and later development of Sjögren's syndrome are in accordance with a registry-based study from Denmark where n=1977 patients had been identified by ICD codes and associations with a history of hospitalizations for infections was investigated [194]. In that study, the number of infections was associated with a dose-dependent risk of Sjögren's syndrome, which is also in agreement with our observations (**Paper II**, Figure 1). Likewise, a small, interview-based study found increased rates of infections requiring hospitalization prior to diagnosis among patients diagnosed according to the AECG criteria compared to controls [195].

Limitations of our study include a lack of data on oral infections, and that we were unable to discriminate between viral and bacterial infections in our analyses. Strengths include that the patients were classified according to the AECG criteria, that we were able to perform analysis stratified by autoantibody status, and the contextually large number of patients.

In all, our study adds to growing evidence to suggest infections as an important environmental factor in the pathogenesis of Sjögren's syndrome. However, no studies have thus far considered potential associations with minor infections that do not require specialized care, and this remains an important query for future studies.

Infections remain a main suspect as trigger of immunopathogenic mechanisms in Sjögren's syndrome. While efforts have been made to identify specific infections that could initiate or propagate disease, no single microbial agent has been convincingly identified and it seems plausible that several different infections may act to stimulate the initiation of autoimmune processes (reviewed by myself and colleagues in [66]). In order to learn more about such microbial triggers, it is of great interest to study serological and cellular responses during infection in patients with an immune system prone to autoimmunity. With this in mind, we designed the studies which resulted in **Paper III** and **IV**, in which we monitor immunologic perturbations following exposure of viral antigens in untreated and HCQ-treated patients with Sjögren's syndrome and SLE.

4.2 CELLULAR AND TRANSCRIPTIONAL PROFILING IN SJÖGREN'S SYNDROME

Alterations in several parts of the cellular immune system are evident in the exocrine tissue pathology of Sjögren's syndrome, with disturbances in T cells, B cells, and glandular cells. Early lymphocytic infiltrates in salivary glands are characterized by a high proportion of T cells, around 80%, and to a lesser extent B cells, although the proportion of B cells increases in advanced lesions [196]. The ratio of $CD4^+/CD8^+$ T cells is highly biased toward $CD4^+$ cells, which display an activated phenotype and produce pro-inflammatory cytokines [197]. Moreover, epithelial cells have been suggested as important mediators of pathology with expression of immune-competent molecules [76], and with the capability of mediating activation and differentiation of T cells [198].

The direct involvement of B cells in the immunopathology of Sjögren's syndrome is apparent from frequent observations of hypergammaglobulinemia, production of autoantibodies and studies showing deranged B cell subpopulations [199]. Furthermore, formation of ectopic GCs can be observed in minor salivary glands of patients and is associated with an increased risk of lymphomagenesis, primarily non-Hodgkin lymphomas of B-cell type [102]. The chemokine CXCL13 and its corresponding receptor CXCR5 are of central importance for the recruitment and organization of B and T cells in the GCs. Indeed, the importance of the CXCR5-CXCL13 axis has been demonstrated in murine models where *CXCR5* deficient mice miss various types of peripheral lymph nodes and the majority of Peyer's patches [200]. Correspondingly, overexpression of CXCL13 in mice results in augmented formation of ectopic lymphoid tissue [201]. In addition, the involvement of *CXCR5* in the pathogenesis of Sjögren's syndrome was further implied in the largest genetic association study to date, in which gene polymorphisms close to the *CXCR5* locus associated with disease [94].

With the aim to better understand mechanisms underlying cellular dysregulation in Sjögren's syndrome, we performed whole transcriptome sequencing of peripheral $CD19^+$ B cells (**Paper VI)** and explored abnormalities of the CXCR5-CXCL13 axis (**Paper VII)**.

4.2.1 The transcriptome of peripheral B cells

To identify differences in gene expression, we performed RNA sequencing of peripheral $CD19^+$ B cells from n=12 patients and n=20 healthy controls. At the time, our study was the first to employ whole transcriptome sequencing on this cell type. For validation of our findings, we employed Nanostring gene expression analysis in a replication cohort consisting of n=16 patients and n=17 controls. All included patients were diagnosed according to the AECG criteria [86]. In order to reduce variability in the cohort, we chose to only include female patients positive for anti-Ro/SSA autoantibodies. Peripheral B cells were isolated using CD19-specific microbeads and purity above 95% of the isolated cells was confirmed by flow cytometry.

4.2.1.1 RNA sequencing of peripheral B cells

In the RNA sequencing dataset, 4047 autosomal genes were found to be differentially regulated, out of which 1826 had upregulated and 2221 had downregulated expression levels comparing patients and controls (**Paper VI**, Supplementary table S2 and S3).

A prominent representation of genes related to IFN responses was noted among the genes with higher expression in patients, both including genes associated with type I IFN responses such as *MX1*, *IFI27*, *IFI44L*, *IFI44*, *OAS1*, and *OAS2* but also genes indicative of responses to type II IFN such as *GBP1*, *GBP*, and *IFI16*. A subsequent gene enrichment analysis could confirm the significant enrichment of genes related to IFN signaling (**Paper VI**, Supplementary Table S6). We thus, for the first time, provided evidence of an IFN signature in peripheral $CD19^+$ B cells, which has thereafter been confirmed by RNA sequencing in two recent studies [202, 203]. Of note, prior to our study, a type I IFN signature in $CD19^+IgD^+$ naïve B cells from untreated patients with Sjögren's syndrome had been described [191]. A type I IFN signature had previously also been detected in salivary gland tissue, whole blood, and in various peripheral blood mononuclear cells [81, 113, 114, 204, 205]. To compare levels of type I IFN-induced gene expression in patients and controls, we calculated IFN scores from expression levels of ISGs, and found these to be higher in patients compared to controls (**Paper VI**, Supplementary figure S3).

Among the downregulated genes, we noted several genes involved in negative regulation of cytokine signaling, including several members of suppressors of cytokine signaling (SOCS) such as *SOCS1* and *SOCS3*, which are negative-feedback inhibitors that act via the JAK/STAT pathway and are important regulators of type I IFN pathway signaling. Other noteworthy regulators with lower expression in patients compared to controls included *PTPN1* which dephosphorylates JAK and TYK kinases, and *TNFAIP3* encoding the protein A20 which is a negative regulator of NF-κB signaling. Interestingly, a germline missense variant of the *TNFAIP3* gene has been associated with lymphoma in Sjögren's syndrome [206], thus further indicating the importance of this gene in B cell dysregulation.

A specific analysis was carried out for genes on the X chromosome among patients and female controls, in which higher expression of *TLR7*, *TLR8* and *Bruton tyrosine kinase* (*BTK*) was noted.

4.2.1.2 Replication of gene expression data

For validation of the results acquired by RNA sequencing in a separate cohort, we employed a Nanostring gene expression panel including 594 genes related to immunology. Partly due to limited overlap, higher expression could be confirmed for 52 genes, and lower expression for 5 genes. Out of the upregulated and replicated genes, *CX3CR1* distinguished itself by displaying the highest fold change. This gene encodes the fractalkine receptor, which has been implicated in B cell lymphomas, including marginal zone lymphomas of mucosa-associated lymphoid tissue (MALT), i.e. the most commonly occurring lymphoma in Sjögren's syndrome [207]. Its ligand, fractalkine, promotes adhesion and migration of inflammatory cells. High protein expression of fractalkine on ductal cells, and of the

fractalkine receptor on inflammatory cells in focal infiltrates, has been demonstrated in salivary glands from patients with Sjögren's syndrome [208].

Higher expression of several genes of the tumor necrosis factor superfamily such as *TNFSF4* encoding OX40L, *TNFSF10* encoding TRAIL, *TNFSF13B* encoding BAFF, and *TNFRSF17* encoding the BAFF receptor could also be validated. Of relevance, OX40L promotes the expansion of T follicular helper cells (Tfh) which are crucial for GC formation [209, 210] and a genetic polymorphism in the *TNFSF4* gene has previously been associated with Sjögren's syndrome [211]. Further, the higher expression of *BAFF* and its receptor's genes fits well with previous data showing that levels of BAFF are increased at the mRNA and protein level in salivary glands, and as circulating protein in patients with Sjögren's syndrome compared to controls, correlating with autoantibody titers and with disease activity [113, 119, 212-215]. Of note, *BAFF* expression can be induced by type I IFN [117], and an association between secretion of BAFF by monocytes and the IFN signature has been demonstrated in Sjögren's syndrome [115]. Together, our data suggest a possible mechanism for ongoing B cell activation through transcriptional dysregulation of *BAFF* and its functionally related genes.

The higher expression of *TLR7* and its downstream signaling molecule *IRF7* were replicated, and increased expression of *IRF7* in circulating $CD19^+$ B cells has subsequently been confirmed in two later studies [202, 203]. Other noteworthy differentially regulated genes included *CCL5* (RANTES) and its receptor *CCR1*, *STAT1*, *STAT2*, and *TNFSF7* also known as CD70.

4.2.1.3 Gene expression in relation to DNA methylation

Gene methylation data were available for co-analysis from a previous study, which had partly included the same patents. A total of 77 genes were shown to be both differentially expressed and differentially methylated, out of which many where ISGs. Of particular interest in the context of **Paper VII**, we could identify hypermethylation in the promotor region of *CXCR5*, which was associated with a lower gene expression in patients compared to controls. The lower expression of *CXCR5* in B cells observed in **Paper VI** is in line with discoveries made in **Paper VII**, namely, that a genetic polymorphism associated with Sjögren's syndrome results in lower expression of *CXCR5* in $CD19^+$ B cells.

In all, we performed the first whole transcriptome sequencing study of peripheral B cells in Sjögren's syndrome and could identify and replicate dysregulated genes participating in several immunological processes. At the time, we were the first to establish a type I and type II IFN signature in $CD19^+$ B cells, underlining the influence of these cytokines on B cell functions. The ongoing immune activation detected in our study strengthens the rationale for drug trials of biologics targeted at B cell inhibition or depletion. Furthermore, our study identified distinct pathways which may be of essential importance in the pathogenesis of Sjögren's syndrome and therefore could constitute targets for future therapeutic intervention.

4.2.2 Detailing the role of chemokine receptor CXCR5

In 2013, Lessard et al. published the first large-scale association study performed in a Caucasian population, in which several genetic variants associated with Sjögren's syndrome were identified [94]. In that study, polymorphisms in the q23.3 locus on chromosome 11 close to the *CXCR5* locus were identified. Intriguingly, CXCR5-CXCL13 and CXCR4-CXCL12 have been suggested as key chemokine axes in the formation of ectopic GCs [96]. Thus, in **Paper VII**, we set out to investigate the CXCR5-CXCL13 axis in Sjögren's syndrome in order to better understand the impact of the identified disease-associated polymorphisms of the *CXCR5* locus.

4.2.2.1 A novel eQTL effect at the CXCR5 gene locus

To comprehend whether gene expression of *CXCR5* was dependent on the genotype in $CD19^+$ B cells, we performed an expression quantitative trait loci (eQTL) analysis on a cohort of healthy individuals originally described by Fairfax et al. [216]. Since the top-associated SNP rs7119038 from the study by Lessard et al. was not available in the Fairfax et al. dataset [94], we instead used a proxy SNP rs4938573 which was in high linkage disequilibrium ($r^2>0.8$) with rs7119038. The analysis revealed that the disease risk allele T resulted in a significantly, albeit fold-change wise moderately lower expression of *CXCR5* in $CD19^+$ B cells.

4.2.2.2 Differential cell population frequencies

To examine frequencies of T and B cell subpopulations expressing CXCR5 and to achieve semi-quantitative values of CXCR5 cell surface expression by measuring median fluorescence intensity (MFI), we performed flow cytometry experiments in a cohort of untreated anti-Ro/SSA positive patients and in healthy controls. We could confirm previous observations in the B cell compartment [199] in the form of lower frequencies of marginal zone (MZ) B cells and tendencies of higher naïve and lower memory B cell proportions in patients compared to controls. Notably, it has been hypothesized that lower frequencies of $CD27^+$ memory cells in patients with Sjögren's syndrome could result from recruitment of these cells to inflamed glandular tissue, or because of exaggerated differentiation into plasma cells [217]. Further, the soluble form of CD27 (sCD27) has been described at higher levels in the circulation of patients compared to controls [218], an observation which is also supported by our data (**Paper III**, Supplementary figure S4A), and sCD27 has been proposed as a marker of abnormal differentiation of memory B cells into plasma cells [217].

In the patients, frequencies of CXCR5 positive cells among total B cells were reduced owing to lower frequencies of CXCR5 positive memory, MZ, and $CD27^-IgD^-$ double negative (DN) cell subsets. Moreover, CXCR5 cell surface expression measured in MFI exhibited a similar tendency of lower expression, which corresponds with the results of the eQTL analysis. For a majority of the patients, we had SNP data for rs4938573 and could thus relate CXCR5 surface expression to genotype. Although no patient was homozygous for C, patients homozygous for the risk allele T had a lower cell surface expression of CXCR5 on $CD19^+CD27^+IgD^-$ memory B cells and a tendency of lower expression in MZ and DN B

cells, indicating that the risk-associated genotype contributes to lower levels of CXCR5 expression.

In the T cell compartment, we noted lower frequencies of CXCR5 positive Th17 cells comparing patients and controls, however, frequencies of Tfh cells were not found to differ. Tfh cells assist in the differentiation of B cells into plasma cells in GCs and have previously been reported at higher frequencies in the circulation of patients with Sjögren's syndrome, also correlating with lymphocytic infiltration of the salivary glands and with disease activity scores [219]. Of note, four of the patients in our study had increased levels of Tfh cells but these patients did not stand out in terms of clinical or histological data. Considering the fact that frequencies of Tfh cells previously have been associated with disease activity, one explanation as to why we did not note differences in frequencies of Tfh cells could be that the patients of our study had relatively low disease activity.

4.2.2.3 Abundance of CXCR5$^+$ cells in target tissue

We hypothesized that the lower abundance of CXCR5 positive cells in the circulation might result from increased homing to the target organ. In line with this hypothesis, we noted higher levels of CXCL13 protein in the circulation of patients compared to controls. Interestingly, higher levels of CXCL13 in the blood of patients with Sjögren's syndrome has been associated with a history of lymphoma [220, 221]. Further, by immunohistochemical staining, we could detect abundant CXCL13 protein expression and higher numbers of CXCR5 positive cells within focal infiltrates and interstitially in salivary glands from patients compared to controls.

In all, we detected lower frequencies of CXCR5 expressing B cell subsets, which also displayed lower cell surface expression of CXCR5 in patients compared to controls. Furthermore, patients had higher levels of CXCL13 in the circulation and abundant CXCL13 protein and high numbers of CXCR5$^+$ cells in the salivary gland tissue. Of note, a recent study found expression of CXCL13 to be induced by type I IFN [222], thus indicating a mechanism whereby high circulating levels in patients may be generated. While the lower CXCR5 expression on cells in patients could partly be explained by an eQTL effect caused by the disease-associated polymorphism, we suggest that these observations likely relate to increased homing of CXCR5$^+$ cells to the target tissue. Our data thus further indicate dysregulation of the CXCR5-CXCL13 axis in Sjögren's syndrome, suggesting that this pathway may be a candidate for therapeutic intervention.

4.3 QUANTIFICATION OF INTERFERON SYSTEM ACTIVITY

Although there is strong support for the involvement of type I IFNs in the immunopathology of systemic autoimmune diseases [223], their roles are highly complex and remain incompletely understood. While IFNs contribute to efficient protection against viral infections, chronic exposure to increased levels of type I IFNs such as in SLE and Sjögren's syndrome is thought to shift the immune system toward pathological functions [223]. In fact, highlighting the complex properties of IFNs, around 3000 genes have been annotated as IFN regulated in the Interferome database [224], with functions including but not limited to anti-

viral and bacterial defense, immune regulation, apoptosis, and cell differentiation. Furthermore, on one hand, type I IFNs may be used to treat MS and certain types of cancer, yet on the other hand, such treatment may cause development of SLE or symptoms of SLE, although often transiently with symptoms that usually fade when treatment is discontinued [45, 159, 225]. In Sjögren's syndrome, an increased type I IFN signature has been associated with anti-Ro/SSA and anti-La/SSB autoantibodies, higher serum IgG titers, lower lymphocyte counts, and higher ESSDAI scores [115, 226-228].

As reviewed in the introduction to this thesis, various reasons make the direct measurement of type I IFNs difficult, and therefore, expression levels of ISGs are usually assessed to calculate mRNA-based IFN scores. Although both type I and type II IFNs have been implicated in the pathogenesis of Sjögren's syndrome [226], a considerable overlap in their induction of ISGs complicate discrete understanding of their specific contribution to the immunopathology of the disease.

4.3.1 Novel methods for quantification of interferon system activity

Standard assessment of IFN system activity relies on RNA samples, which also need to be well preserved since RNA will degrade over time. However, plasma, serum, and DNA samples are often more available in historical cohorts and large research studies. With this in mind, we therefore set out to find alternative markers for IFN system activity, which resulted in **Paper V**.

4.3.1.1 Quantification of IFN scores based on expression of ISGs

To establish gene expression-based IFN scores to be used as reference, we measured the expression of ISGs by qPCR and by Nanostring technology in peripheral blood monocytes and B cells. As could be expected, we found high correlation between IFN scores measured by qPCR and by Nanostring, confirming previous observations that Nanostring may be used for accurate and easy quantification of IFN scores [229, 230]. In fact, Nanostring technology can be used directly on cell lysates, thus eliminating cumbersome steps of RNA isolation and amplification, which is advantageous for clinical application. Further, we could demonstrate correlations between mRNA-based IFN scores in simultaneously sampled monocytes and B cells, which had not been specifically shown in Sjögren's syndrome before, thus indicating that either of these cell types may be used for assessment of type I IFN scores with similar results. Indeed, higher IFN scores has previously been described in many cell types and in the target tissue in Sjögren's syndrome patients [81, 113, 115, 226], showing that the effect of type I IFNs is widespread and systemic. Induced transcription of ISGs by type I IFNs depend on the cell surface expression of IFNAR as well as the expression of downstream signaling components. In future studies in Sjögren's syndrome, it would be interesting to determine whether certain cells types are more sensitive to changes in IFN levels, which could potentially be of added value in clinical studies where the type I IFN score is used as a biomarker.

4.3.1.2 Establishing the pIFN score

To establish a pIFN score, we employed PEA technology in plasma and serum using a panel of 92 proteins selected for their relevance in immunology. In our analysis, a combined score calculated from relative levels of CXCL10, CXCL9, and the soluble form om PD-1 (sPD-1) was found to correlate the best with the mRNA-based IFN scores. It is possible that screening other PEA panels or proteins would bring about other or more relevant IFN activity markers, but in our dataset the addition of further proteins did not improve performance of the score. Although the proteins used for calculation of the pIFN score had been identified based on the fact that their corresponding mRNA expression is type I IFN induced [224], we hesitate to claim that the pIFN score would be specific only for type I IFN activity, partly since both CXCL10 and CXCL9 are known to be induced also by IFN-γ. Other studies have proposed soluble or cell surface bound proteins as surrogate markers of IFN system activity in systemic autoimmune diseases [227, 231-234], however, keeping in mind the many subtypes of type I IFN and that the profile of ISGs may differ between individuals, we reasoned that a pIFN combining levels of several proteins would be more stable than a single protein marker alone.

To understand whether the score would perform similarly in serum as in plasma, we measured the pIFN score in simultaneously collected serum and plasma samples. Indeed, we found that pIFN scores correlated well between serum and plasma, thus further supporting its usefulness. The cohort used to establish the pIFN score only encompassed anti-Ro/SSA positive patients. Therefore, to determine pIFN score levels also in autoantibody negative patients, we measured serum pIFN scores in a large cohort encompassing both anti-Ro/SSA positive patients, and patients negative for both anti-Ro/SSA and anti-La/SSB. We found the pIFN score to be the highest in anti-Ro/SSA positive patients, which is in accordance with previous reports [115, 226], however, the score was significantly higher also in autoantibody negative patients compared to controls. This observation is particularly intriguing considering that proposed models of type I IFN production in Sjögren's syndrome generally describe a mechanism through which autoantibodies form RNA-containing immune complexes which undergo FcgRIIa-mediated endocytosis by pDCs whereby endosomal TLRs are stimulated, thus resulting in production of type I IFN [112]. However, although pDCs have long been viewed as main producers of type I IFN in autoimmune diseases [235], this notion was recently challenged in a publication showing that pDC from patients with Sjögren's syndrome and SLE may have lost their capacity for TLR-mediated cytokine production and that they have reduced telomere length, together with a transcriptional signature indicative of cellular stress and senescence [236]. To my knowledge, levels of type I IFN activity in autoantibody negative patients with Sjögren's syndrome compared to controls has not been specifically studied. Unfortunately, we do not have RNA samples to generate mRNA-based IFN scores in the autoantibody negative patients of our cohort, and I therefore cannot assess whether the increased levels of the pIFN score in these patients correlates with expression of ISGs, or if discrete mechanisms are causing the increased levels of the proteins.

The possibility to assess IFN system activation in serum and plasma samples enables new insights in historical cohort for which only such samples have been stored. This is the case for many older biobanks. However, the effect of storage conditions and time of storage on the pIFN should be evaluated in future studies.

4.3.1.3 Establishing the DNAm IFN score

Based on our observations of hypomethylation of ISGs in **Paper VI**, we hypothesized that an IFN activity score similar to the pIFN score could be established based on levels of DNA methylation. To generate the DNAm IFN score, we assessed correlation of CpG methylation levels at type I IFN induced genes [224] to RNA sequencing-based mRNA scores in B cells from a separate cohort of patients and controls. Reassuringly, the assessment of ISG expression by qPCR compared to RNA sequencing has been found to yield comparable and consistent results [237]. We calculated the DNAm IFN score based on beta values of CpG sites at three genes, and the score was found to correlate well with the mRNA-based scores and to achieve high concordance rate (97.2%) with the mRNA-based score when classifying individuals as having positive or negative IFN scores. In a separate cohort, additional assessment of the DNAm IFN score in whole blood confirmed higher scores in anti-Ro/SSA positive patients compared to controls, and likewise, significantly higher scores in autoantibody negative patients compared to controls, which further substantiates the corresponding observations when employing the pIFN score. The ability to assess both genotype and phenotype, i.e. IFN system activation, in DNA samples opens up great possibilities, not least in large cohort studies such as GWAS studies for which DNA may be the only biologic material available for analysis.

The ability to determine IFN scores by measurement of gene expression, protein, and DNA methylation levels generates new questions regarding the kinetics of the various scores. Indeed, DNA methylation patterns are mitotically heritable at the cellular level and are thought to be relatively stable over time, yet possibilities of methylation changes in response to external stimuli are still maintained [238]. Consequently, it could possibly be so, that the DNAm IFN score is more stable over time, while mRNA and protein-based scores are more dynamic and subject to faster changes. This line of thought would suggest that the DNAm IFN score could be better at accurately classifying patients and controls, whereas the pIFN score may constitute a better biomarker e.g., of responses to treatment. In our study, we did not have the possibility to examine such kinetics of the different scores, which is something I would like to assess in futures studies. I am especially interested in the temporal circumstances regarding hypomethylation of ISGs and whether a high DNAm IFN score perhaps could identify individuals at increased risk of developing systemic autoimmunity. However, studies indicating that gene expression precedes hypomethylation would speak against such a scenario [239].

Limitations of our study relate to the fact that we did not have the possibility to assess the pIFN and DNAm IFN scores in the same samples nor in other infectious or rheumatic diseases, which would have been of added value.

4.3.1.4 Assessing IFN system activity in systemic autoimmune diseases

The complex involvement of type I IFNs in systemic autoimmune disease and their widespread effects in the body are still being untangled. However, in a recent phase III trial of the anti-IFN receptor monoclonal antibody anifrolumab, a higher frequency of response measured by British Isles Lupus Assessment Group-based Composite Lupus Assessment (BICLA) at week 52 was noted in the treated compared to the placebo group (47.8% versus

31.5%), thus validating the type I IFN pathway as an effective treatment target [160]. As intended, the drug rapidly neutralized the IFN signature in the actively treated patient group. However, further adding to the complexity of the IFN system in Sjögren's syndrome, a recent phase II trial of RSLV-132, an RNase Fc fusion protein, revealed that treatment with this drug counterintuitively resulted in upregulation instead of downregulation of IFN-inducible genes, while at the same time improving symptoms of severe fatigue [240]. The potential of targeting IFN signaling in the treatment of Sjögren's syndrome is still attractive considering the encouraging evidence for efficacy of anifrolumab in SLE [160]. In future trials of such drugs, various measures of IFN system activity could be of central importance, for which the methods presented in **Paper V** can be considered.

Bearing in mind the potential differences in kinetics of the various IFN scores, it would be very interesting to assess all of these scores simultaneously e.g., in the clinical trials evaluating anifrolumab. Does the blocking of the type I IFN receptor effect the DNA methylation profile at IFN inducible genes? If so, when does this change occur and how long does it persist after discontinuation of treatment? Such a study could provide further insights into the dysregulation of type I IFN signaling in systemic autoimmunity.

Together, our study provides new tools for the assessment of IFN activity, which are especially valuable in instances where RNA samples are not available. The methods we present may not only be of value in large studies where only plasma/serum and DNA has been collected, but their employment could potentially lead to a better understanding the kinetics of IFN dysregulation in systemic autoimmunity.

4.4 MICROBIAL ANTIGEN EXPOSURE IN SYSTEMIC AUTOIMMUNITY

The hypothesis that infections may trigger or propagate systemic autoimmunity is long-standing, and we have presented additional evidence to support this model in **Paper II** of this thesis. However, efforts to identify specific infectious agents causing development of Sjögren's syndrome, or the immunologically related disease SLE, have not been very successful [66, 241]. An explanation for this could be that several different infectious agents have the potential of provoking autoimmune processes in genetically predisposed individuals. Hypothetically, infections could act in a non-specific manner to propagate and amplify otherwise quiescent deficiencies in central or peripheral tolerance mechanisms, which may already be ongoing in susceptible individuals. However, to detect such defective pathways in humans would require the *in vivo* study of immune mechanisms during infections, which is logistically challenging when it comes to spontaneously occurring infections, and ethically problematic if infections would need to be induced. In our efforts to better understand immunological perturbations during encounters with microbial antigens in the context of autoimmunity, we therefore monitored responses following vaccinations, which are safe and recommended for patients with rheumatic diseases [169]. We specifically studied patients with no or little treatment in order to understand processes transpiring in an immune system unmanipulated by intense treatment.

Prior to this thesis, Brauner et al. had reported that adjuvanted anti-H1N1 vaccination in untreated patients with Sjögren's syndrome resulted in higher vaccine-specific antibody titers

compared to controls, together with increased autoantibody titers in the patients [191]. However, the vaccine used in that study contained the adjuvant squalene and it remained unknown whether non-adjuvanted vaccination would result in similar observations. Furthermore, initial innate responses which may underlie production of higher antibody levels had not been assessed.

Therefore, to identify differences in serological and cellular responses following non-adjuvanted viral antigen exposure, we monitored untreated and HCQ-treated female patients with Sjögren's syndrome and SLE, and age-matched healthy controls during non-adjuvanted trivalent seasonal influenza vaccination in **Paper III** and **Paper IV**.

4.4.1 Augmented responses against viral antigens

A main finding of our studies was that untreated patients with Sjögren's syndrome and patients with SLE receiving no or light treatment responded to viral antigens with higher vaccine-specific antibody titers compared to healthy controls. In SLE, patients receiving HCQ with or without low-dose prednisolone developed similar titers as untreated patients, whereas the HCQ-treated group of patients with Sjögren's syndrome had a non-significant tendency to respond with lower titers compared to untreated patients, yet still with non-significantly higher titers than controls.

Of note, we aimed to assess an overall B cell reactivity in our studies and therefore primarily focused on measuring all antibodies targeted against the viral antigens, and not only neutralizing antibodies, which are commonly measured when assessing vaccine responses in terms of seroconversion and seroprotection rates. Furthermore, our statistical assessment of antibody production focused on the relative change, and not the absolute levels, in antibody titers. Whereas a previous study, as mentioned above, supports our observations in Sjögren's syndrome [191], studies in SLE have mostly reported lower frequencies of protective antibodies in patients compared to controls [168, 242, 243], while untreated and HCQ-treated SLE patients seem to attain similar seroprotection frequencies as controls [168, 244]. However, importantly, these studies have generally assessed neutralizing antibodies and not, as in our studies, all antibodies mounted against the foreign antigens. Consequently, our results could be interpreted as patients producing an overall larger number of antibodies targeting the vaccine, whereas the effects of neutralizing antibodies may not differ compared to controls. Nevertheless, in **Paper III**, neutralizing antibodies against hemagglutinin measured by hemagglutination inhibition assay against the H3N2 strain were indeed higher in patients compared to controls, but there were no differences for the other strains.

A possible explanation for the higher antibody production could be that autoimmune patients, due to defects in tolerance mechanisms, have a larger pool of naïve B cells with a higher number of potential reactivities. Another possible explanation could be that due to ubiquitous autoimmune processes, patients have a larger amount of lymphoid tissue e.g., GCs and will therefore mount amplified antibody responses. Indeed, patients with Sjögren's syndrome and SLE have high levels of the GC-associated chemokine CXCL13 in the circulation [221, 245, 246], and frequently form ectopic GCs in which high numbers of Tfh cells critical for B cell antibody responses are present. An additional explanation could be

that B cells in patients are in a state of general hyperactivity caused by chronic stimulation from type I IFNs and associated high levels of the B cell simulating cytokine BAFF. Moreover, another important factor is the possible association between disease risk-associated HLA haplotypes and development of higher antibody responses. Ideally, the healthy control group could be genetically matched to the patient group in terms of risk-allele carriage in order to account for this factor, which we were unfortunately not able to do in our studies. In fact, various HLA class II haplotypes have been associated with differences in serological responses against influenza antigens [247-249]. Therefore, future studies dissecting responsiveness to viral antigens in patients with systemic autoimmunity should take genetic factors such as HLA into consideration.

In order to reduce inter-individual variability and to avoid interference by immune-modulatory drugs, we only studied patients with Sjögren's syndrome positive for anti-Ro/SSA autoantibodies, and patients with SLE receiving no or light treatment i.e., with low-active disease. Therefore, our results may not be generally applicable to the patient groups as a whole. However, in SLE, we believe that studying this group of patients gives insight into processes that would also be true for patients with higher disease activity, had they not been treated, although this remains to be demonstrated.

4.4.1.1 Autoantibody titers following vaccination

Bystander activation of autoreactive B cells has been proposed as a mechanism whereby infections could trigger autoimmunity. In a previous study, increased autoantibody titers following vaccination were observed in untreated patients with Sjögren's syndrome [191]. Therefore, to assess potential non-specific activation of B cells in our studies, we measured autoantibody titers in patients. In Sjögren's syndrome, anti-Ro52 autoantibodies were found to increase in untreated but not in HCQ-treated patients. However, in patients with SLE, who present with a broader range of autoantibodies, titers of several autoantibodies were analyzed yet none were found to be affected in neither untreated nor in patients on light treatment. This may be related to the power of the study since there were relatively few patients in each group when stratified according to autoantibody specificities. The increase in anti-Ro52 autoantibodies in Sjögren's syndrome patients are in line with the study by Brauner et al., however, the relative change in **Paper III** was less than that observed for a squalene-adjuvanted vaccine [191]. In SLE, previous studies present diverging data, with some indicating increased titers of autoantibodies following influenza vaccination [250, 251], and others not [252, 253].

It is interesting that treatment with HCQ seemed to counteract production of autoantibodies in patients with Sjögren's syndrome, an observation which corresponds to the non-significant tendency for lower production of vaccine-specific antibodies in those patients. Hydroxychloroquine partly acts by inhibiting endosomal TLR signaling [254, 255], and treatment with HCQ has been reported to lower the expression of ISGs in patients with Sjögren's syndrome [256]. Correspondingly, naïve B cells from patients treated with HCQ have a lower propensity to differentiate into antibody secreting plasmablasts upon endosomal TLR ligation [191]. Although the largest clinical trial to date did not demonstrate efficacy for

HCQ treatment in terms of decreasing symptoms in Sjögren's syndrome [127], our data suggest potential protective effects in terms of suppressed autoantibody production.

4.4.1.2 Vaccinations are well-tolerated

In our studies, vaccination against influenza was not associated with any exacerbations of underlying disease and patient-reported symptoms remained unchanged both in patients with Sjögren's syndrome and SLE during a follow-up period of 90 days. Although our studies were not designed to assess safety, these observations confirm previous reports that vaccination does not aggravate clinical symptoms of rheumatic diseases [257]. While there have been case reports of disease development or flares of Sjögren's syndrome and SLE following vaccination, literature reviews have not corroborated such observations [165, 257]. Rather, it has been concluded that the advantages of vaccination in rheumatic patients by far outweigh potential risks. Together, our data support the current recommendations by EULAR i.e., that non-adjuvanted influenza vaccination appears safe and should be encouraged for patients with Sjögren's syndrome and SLE.

In conclusion, augmented antibody responses toward viral antigens in the two diseases suggest a state of hyperreactivity in B cells and further signifies viral infections as an important environmental risk factor for systemic autoimmunity. Furthermore, our data for Sjögren's syndrome indicate a mechanism whereby viral antigens trigger bystander activation of autoreactive cells, which is in concordance with our observations in **Paper II,** in which a history of infections more closely associated with development of autoantibody positive disease. We also conclude that non-adjuvanted, compared to squalene adjuvanted, vaccines may be preferable when vaccinating untreated patients with Sjögren's syndrome. Finally, our data support that influenza vaccination does not cause increased autoantibody production in patients with low-active SLE.

4.4.2 A relationship between type I IFN system and antibody responses

Monocytes are important effector cells at the interface of innate and adaptive immunity. In Sjögren's syndrome compared to controls, a higher monocyte expression of type I ISGs together with higher expression of BAFF, and TLR7 and its downstream signaling molecules had previously been reported [115, 205].

To better understand which factors that may underlie augmented serological responses, we therefore performed gene expression analysis in $CD14^+$ monocytes from peripheral blood. In **Paper III**, the pre-vaccination transcriptional signature assessed by Nanostring was dominated by a type I IFN signature, and a higher type I IFN system activation was found to associate with a higher vaccine-specific antibody response at day 28. Based on these observations, we opted for a qPCR-based IFN score in **Paper IV** and observed a corresponding relationship between IFN score and vaccine-specific antibody titers that developed in patients with SLE. Although we had not observed any changes in autoantibody titers in patients with SLE after vaccination, we found that the number of autoantibody specificities was highly positively correlated with the IFN score.

In all, we observed that higher pre-vaccination type I IFN scores in monocytes associate with augmented production of vaccine-specific antibodies, thus supporting proposed models in which high type I IFN activation promotes B cell activation and antibody production in systemic autoimmunity [120]. Our data are in line with experiments showing that pre-stimulation of naïve B cells from healthy subjects with IFN-α enhances plasmablast differentiation and class switch [191]. Interestingly, it was recently reported that a gene signature composed of ten IFN-regulated genes was useful for predicting both antibody responses to influenza vaccination in healthy subjects as well as reflecting the magnitude of plasmablast-associated disease flares in patients with SLE [258, 259]. Although increased IFN scores have been found to associate with higher disease activity in both Sjögren's syndrome and in SLE [171, 227, 228], the observations made in healthy subjects therefore suggest that it may in fact be type I IFN, and not disease activity itself, which associates with higher antibody production.

4.4.2.1 The proteome in relation to vaccination

To detect perturbations in the blood proteome following viral antigen exposure, we measured the levels of 92 proteins in plasma before and 24 hours after vaccination (**Paper III)**. In our analysis, we specifically aimed to identify proteins displaying significantly different responses comparing patients and controls and found differential regulation of the levels of the monocyte-attracting chemokine CCL7. Also, in line with the observations of increase in type I IFN scores in both patients and controls (**Paper III**, Figure 3C), the IFN-induced proteins CXCL10 and CXCL9 were identified in the analysis, however, these proteins displayed similar regulation in both patients and controls. CCL7 is involved in trafficking of monocytes, thus suggesting differences in the recruitment of monocytes following vaccination, possibly to the site of injection. Although the proteome screen was comprehensive, we detected differential regulation of relatively few proteins, which likely relates to the fact that a later time point would have been better for assessment of changes in the proteome, since production of such proteins generally could be expected to take more than 24 hours. With this in mind, we focused our resources on only analyzing the proteome prior to vaccination in **Paper IV**.

To assess whether blood proteins could be useful in predicting antibody responses to vaccination, we analyzed pre-vaccination protein levels in relation to vaccine-specific antibody titers at one month. The proteins with the strongest correlation were sPD-1 in Sjögren's syndrome and CCL3 in SLE, which notably are both type I IFN regulated proteins at the mRNA level [224]. Indeed, sPD-1 was used to calculate the pIFN score in **Paper V** and CCL3 has previously been suggested as a marker of type I IFN activation in SLE [260]. Further supporting a central role for PD-1 in the pathogenesis of autoimmune disease are reports of patients developing manifestations of systemic autoimmune disease following checkpoint inhibition of the PD-1/PD-L1 pathway during treatment for cancer [19]. Although the top proteins correlating with antibody titers at day 28 differed between the two diseases, the enrichment of IFN-regulated proteins was striking in both studies.

Of note, many of the proteins detected at significantly differential levels comparing patients and controls (**Paper III**, Supplementary figure S4A; **Paper IV**, Table 2) are

generally thought of as membrane-bound proteins. The fact that we detect these proteins in the circulation suggests that they have either been shed from the membrane, or that they have been actively secreted. The soluble form of these proteins may have properties to inhibit or intensify their signaling pathways. For example, sPD-1 has been proposed to block the PD-1/PD-Ls pathway, thereby promoting T cell responses [261]. Therefore, the detection of soluble forms of classical membrane-bound proteins requires further studies in order to understand their function and possible contribution to autoimmune pathogenesis.

Together, we observe markers of type I IFN activation also at the protein level and their association with higher production of antibodies after viral antigen exposure further imply type I IFNs to be of central importance in generation of augmented humoral responses. Several differentially abundant proteins identified in our blood proteome screens, which have not previously been reported, warrant closer investigation and replication in separate cohorts.

4.4.3 Differences in early transcriptional events suggest roles for NF-κB

Whereas previous studies have detailed steady-state transcriptional profiles of various cell types in Sjögren's syndrome, no studies prior to this thesis had specifically examined transcriptional responses following exposure to microbial antigens.

To comprehend potential differences in transcriptional changes induced by viral antigens comparing patients and controls, we analyzed changes in gene expression from before and 24 hours after vaccination in monocytes. We found that the most significant gene with a simultaneous relative increase in Sjögren's syndrome patients compared to controls was the *IKBKG* gene encoding the protein NF-κB-essential modulator (NEMO). Moreover, we observed that the induced expression of NEMO, as well as of a summarized NF-κB activation score, correlated with vaccine-specific IgG titers at day 28.

The NEMO protein is a part of the IKK complex which acts to activate NF-κB gene transcription by phosphorylation of the inhibitory IκBα (**Figure 1**). While this protein has not previously been studied in Sjögren's syndrome, human deficiencies of NEMO have been associated with the disease incontinentia pigmenti [262], as well as other immunodeficiencies [263], and mutations have been shown to result in defect NF-kB activation and impaired responses to TLR agonists [264, 265]. Intriguingly, and in line with our data, monocytes in Sjögren's syndrome were previously shown to express lower levels of IκBα compared to controls [266], and increased gene expression of the NF-κB transcription factors *NFKB1* and *NFKB2* [267]. In addition, identification of genome wide association with the gene *TNFAIP3 interacting protein 1* (*TNIP1*), which encodes an A20-bining protein [94], further suggests that dysregulated activation of NF-κB signaling may be important in the immunopathogenesis of Sjögren's syndrome.

Altogether, our data indicate that dysregulated activation of NF-κB in monocytes may, at least in part, underlie higher serological responses in patients. The suggestive importance of NEMO in the pathogenesis of Sjögren's syndrome should be studied further.

4.4.4 Potential impact of IFN-targeted treatment

To conclude, in our studies of Sjögren's syndrome and SLE, we observed strong indications that type I IFNs exert influence on serological responses to viral antigens, which is in line with previous *in vitro* experiments showing that type I IFN primes naïve B cells for higher plasmablast differentiation and immunoglobulin production [191]. Our data fit well in proposed models where type I IFN leads to production of the B cell stimulating cytokine BAFF, thereby causing increased B cell differentiation and antibody production. Furthermore, our data imply the importance of dysregulated NF-κB signaling in monocytes in the generation of higher serological responses in Sjögren's syndrome. Altogether, our data add to the understanding of immune reactions toward microbial antigens in autoimmune immune systems and imply infections as an important environmental risk factor for autoimmune disease.

A major challenge, however, in identifying relevant pathways in these diseases are the widespread effects of type I IFNs, which have major influence both on gene transcription as well as on blood proteome signatures, thus making it difficult to detect other dysregulated pathways which could be of key importance. To tackle this, it will be especially interesting to study responses to microbial antigens in patients who receive treatment with anti-IFN targeted drugs such as anifrolumab, through which the type I IFN signature is rapidly normalized [160]. Would such treatment lower antibody responses in patients to the level of controls? Would it perhaps significantly impair vaccination responses? Indeed, vaccination leads to type I IFN responses also in healthy subjects (**Paper III**, Figure 3C), which are likely important for generation of adequate serological responses. Upon normalization of the type I IFN signature by anifrolumab in patients, other important pathways may emerge more clearly when they are not masked by otherwise ubiquitously present type I IFN signature. Future studies in patients receiving anti-IFN targeted drugs could therefore be of great value.

5 CONCLUDING REMARKS

In this thesis, we employed different approaches and methodologies to better characterize exogenous and endogenous factors of importance in the immunopathology of Sjögren's syndrome and the related autoimmune disease SLE.

We observed that previous and current cigarette smoking was less frequent among Sjögren's syndrome cases compared to controls. This was related to a decline in smoking exposure among cases starting approximately 35 years prior to diagnosis, which was in part explained by a higher probability of smoking discontinuation among cases compared to controls. We therefore conclude that smoking does not appear to be a risk factor for development of Sjögren's syndrome. However, since disease associated dryness may influence behavior, we aim to more closely define exposure to smoking in incident cases in the future. Moreover, we identified that a history of infections is a risk factor for development of Sjögren's syndrome, with higher risk estimates for anti-Ro/SSA and anti-La/SSB positive disease. Thus, our data add to growing evidence indicating infections as an important environmental risk factor for Sjögren's syndrome. Future studies should assess the influence also of minor infections not requiring specialized care, and risk factor studies should in general employ careful patient stratification based on clinical symptoms, disease manifestations, autoantibody status, as well as genetics.

We observed that percentages of $CXCR5^+$ B cells in the circulation and their surface expression of CXCR5 is lower in patients with Sjögren's syndrome compared to controls. At the same time, the chemokine ligand CXCL13 is increased in plasma and high numbers of $CXCR5^+$ cells are present in salivary glands. This could be explained by increased homing of B cells with high cell surface expression of CXCR5 to the autoimmune target tissue. Furthermore, we found extensive differences in the transcriptome of circulating $CD19^+$ B cells comparing patients with Sjögren's syndrome and controls, including prominent type I and type II IFN signatures in patients. Our results add to the understanding of B cell dysregulation and identify potential therapeutic targets warranting further study.

We present two novel methods which can be employed for quantification of IFN system activation using serum/plasma or DNA samples. These methods are especially valuable in studies where RNA samples have not been collected. An interesting question for future studies would be the dynamics of the IFN signature at the mRNA, protein, and DNA methylation level, especially considering the emerging anti-IFN therapies.

In our studies, untreated patients with Sjögren's syndrome and patients with SLE receiving no or light treatment responded to viral antigen exposure with higher vaccine-specific antibody titers compared to controls, thus mirroring a B cell hyperreactivity. A higher serological response was related to increased markers of activation of the type I IFN system prior to vaccination, and in Sjögren's syndrome a higher response was also linked to dysregulated NF-κB signaling in monocytes. These observations further elucidate mechanisms through which microbial triggers affect autoimmunity-prone immune systems.

Taken together, the data presented in this thesis expand the current understanding of the autoimmune pathogenesis of primary Sjögren's syndrome. A summary of the factors studied in this thesis are presented in the figure below (**Figure 5**).

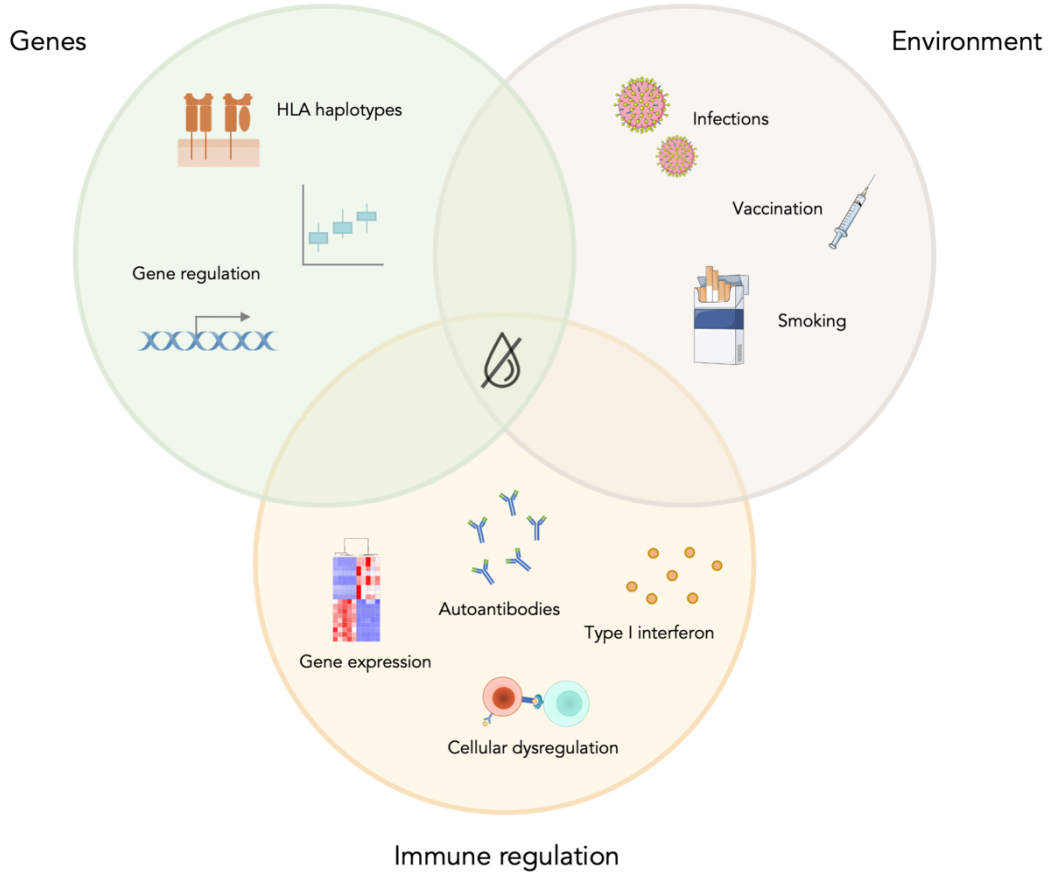

***Figure 5**. Summary of factors studied in this thesis. The figure was created using images from Servier Medical Art by Servier (https://smart.servier.com).*

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