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RISK FACTORS AND COMORBIDITY IN PRIMARY SJÖGREN'S SYNDROME

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Risk factors and comorbidity in primary Sjögren's syndrome

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The public defense will take place at the Center for Molecular Medicine lecture hall, at the Karolinska University Hospital, Solna, June 11th, 2021 at 9:00.

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Difficult to see. Always in motion is the future.

– Yoda

ABSTRACT

Primary Sjögren's syndrome is an autoimmune disease, in which the immune system targets the exocrine glands. The disease is characterized by inflammation and dysfunction of the salivary and lacrimal glands, leading to dry eyes and mouth.

The etiopathogenesis of autoimmune diseases is not entirely known. Genetic factors, primarily relating to the immune system, are central in the development of disease. In Sjögren's syndrome, such genetic variations are associated with the production of autoantibodies to the Ro/SSA and La/SSB autoantigens and a more aggressive course of the disease. However, environmental factors are also involved in the development of autoimmunity. Viruses, smoking, alcohol and radiation include some of the more frequently proposed risk factors, although a causal relationship remains to be been proven.

To expand the current understanding of environmental risk factors, the relationship between exposure to infections and smoking and the development of Sjögren's syndrome were investigated in **Study I** and **Study II**, respectively. A clear association between infections and subsequent development of Sjögren's syndrome was observed, as a history of infections was significantly more prevalent in individuals with Sjögren's syndrome compared to controls from the general population. Notably, this association was even more prominent in patients who developed Ro/SSA and La/SSB autoantibodies. Exposure to smoking could however not be linked to an increased risk of the disease, despite the well-known association with development of other rheumatic diseases. Rather, we observed that individuals who developed Sjögren's syndrome were more prone to stop smoking during the decades preceding diagnosis. This finding may indicate that the appearance of very early symptoms of the disease leads to the discontinuation of smoking.

Sjögren's syndrome is a systemic disease, and may cause adverse events in various organ systems. The risk of cardiovascular and hematological disease in patients with Sjögren's syndrome was analyzed using the Swedish national health-care registers in **Study III** and **Study IV**, respectively. Compared to the general population, individuals with Sjögren's syndrome had a significantly increased risk of myocardial infarction, cerebral infarction, and venous thromboembolism. Moreover, the results indicate that Ro/SSA and La/SSB autoantibodies demark the subgroup of patients with the highest risk of cardiovascular comorbidity. Similarly, an increased risk of multiple myeloma was observed in patients with Sjögren's syndrome, which was confined to individuals with Ro/SSA and La/SSB autoantibodies.

Long-term outcomes for individuals with congenital heart block, which may develop in fetuses whose mothers carry Ro/SSA autoantibodies, were analyzed in **Study V**. The results indicate that these individuals have an increased risk of cardiovascular complications, and also illnesses related to infections and chronic inflammation, suggesting that a systematic follow-up would benefit these patients.

In conclusion, the findings indicate that infections contribute to the development of Sjögren's syndrome. Furthermore, the presence or absence of Ro/SSA and La/SSB autoantibodies discriminate between two distinct patient subgroups, and is a useful parameter for predicting the risk of comorbidity. Lastly, the findings reveal the risk of long-term complications in patients with congenital heart block.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Sjögrens syndrom är en autoimmun sjukdom där kroppens immunförsvar angriper kroppens körtlar, företrädesvis saliv- och tårkörtlar. Dessa blir inflammerade med sänkt funktion, med torrhet i mun och ögon som följd.

Orsaken till varför autoimmun sjukdom utvecklas är ofullständigt känd. Det står dock klart genetiska faktorer, främst relaterade till immunförsvaret, förklarar en del av risken för sjukdom. Hos patienter med Sjögrens syndrom predisponerar sådana genetiska faktorer till bildningen av autoantikroppar mot Ro/SSA och La/SSB, vilka finns närvarande hos cirka två tredjedelar av patienterna och associerar till en svårare sjukdom. Även miljörelaterade faktorer tycks inverka på utvecklingen av autoimmun sjukdom. Virus, rökning, alkohol och strålning tillhör några av de mer frekvent framförda riskfaktorerna, även om ett fullständigt orsakssamband inte kunnat påvisas.

För att utöka kunskapen kring rollen hos miljörelaterade riskfaktorer, undersöktes i **Studie I** och **Studie II** sambandet mellan exponering för infektioner respektive rökning och utvecklingen av Sjögrens syndrom. Ett tydligt samband mellan infektioner och utvecklingen av sjukdomen kunde påvisas, vilket dessutom var mer påtagligt bland individer som utvecklade antikroppar mot Ro/SSA och La/SSB. Ett samband mellan rökning och risk Sjögrens syndrom kunde däremot inte påvisas. Däremot antydde observerade mönster av rökning att sjukdomen börjar att utvecklas flera decennier innan individerna erhåller sin diagnos, då individer som utvecklade sjukdomen uppvisade en ökad benägenhet att sluta röka.

Det inflammatoriska förloppet vid Sjögrens syndrom är inte begränsat till kroppens körtelstrukturer. Cirka en tredjedel av patienterna erfar systemiska symtom som engagerar olika organsystem såsom lungor, hud, leder och nervsystem. I **Studie III** och **Studie IV** analyserades risken för kardiovaskulär respektive hematologisk sjuklighet. I dessa påvisades att risken för sådan samsjuklighet är väsentligt högre hos patienter med Sjögrens syndrom än hos befolkningen i stort. Dessutom indikerar resultaten att patienter med autoantikroppar mot Ro/SSA och La/SSB är den subgrupp av individer med Sjögrens syndrom som löper högst risk för kardiovaskulär sjukdom i from av proppbildning i vener, liksom risk för cancersjukdomen multipelt myelom.

I **Studie V** analyserades sjuklighet hos individer med medfött hjärtblock, vilket kan utvecklas hos foster där modern bär autoantikroppar mot Ro/SSA. Fynden från denna studie antyder att dessa individer löper en ökad risk för framtida kardiovaskulär sjuklighet, men även en ökad risk för infektioner och inflammatoriska sjukdomar.

Sammanfattningsvis antyder resultaten att infektioner ökar risken för att utveckla Sjögrens syndrom, samt att närvaron av antikroppar mot Ro/SSA och La/SSB särskiljer mellan två olika patientgrupper och kan användas för att prediktera risken för komplikationer. Avhandlingen har även bidragit till kunskapen om långtidskomplikationer hos individer med medfött hjärtblock.

LIST OF SCIENTIFIC PAPERS

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

I. **Infections increase the risk of developing Sjögren's syndrome**
Johannes Mofors, Elizabeth V. Arkema, Albin Björk, Linnea Westermark, Marika Kvarnström, Helena Forsblad-d'Elia, Sara Magnusson Bucher, Per Eriksson, Thomas Mandl, Gunnel Nordmark, Marie Wahren-Herlenius
J Intern Med. 2019 Jun;285(6):670-680

II. **Cigarette smoking patterns preceding primary Sjögren's syndrome**
Johannes Mofors*, Albin Björk*, Elina Richardsdotter Andersson, Marika Kvarnström, Helena Forsblad-d'Elia, Sara Magnusson Bucher, Leonid Padyukov, Ingrid Kockum, Jan Hillert, Per Eriksson, Thomas Mandl, Gunnel Nordmark, Lars Alfredsson, Marie Wahren-Herlenius
RMD Open. 2020 Sep;6(3):e001402

III. **Concomitant Ro/SSA and La/SSB antibodies are biomarkers for the risk of venous thromboembolism and cerebral infarction in primary Sjögren's syndrome**
Johannes Mofors, Marie Holmqvist, Linnea Westermark, Albin Björk, Marika Kvarnström, Helena Forsblad-d'Elia, Sara Magnusson Bucher, Per Eriksson, Elke Theander, Thomas Mandl, Marie Wahren-Herlenius, Gunnel Nordmark
J Intern Med. 2019 Oct;286(4):458-468

IV. **Increased risk of multiple myeloma in primary Sjögren's syndrome is limited to individuals with Ro/SSA and La/SSB autoantibodies**
Johannes Mofors, Albin Björk, Karin E. Smedby, Marika Kvarnström, Helena Forsblad-d'Elia, Sara Magnusson Bucher, Per Eriksson, Thomas Mandl, Eva Baecklund, Gunnel Nordmark, Marie Wahren-Herlenius
Ann Rheum Dis. 2020 Feb;79(2):307-308

V. **Comorbidity and long-term outcome in patients with congenital heart block and their siblings exposed to Ro/SSA autoantibodies in utero**
Johannes Mofors, Håkan Eliasson, Aurélie Ambrosi, Stina Salomonsson, Amanda Skog Andreasson, Michael Fored, Anders Ekbom, Gunnar Bergman, Sven-Erik Sonesson, Marie Wahren-Herlenius
Ann Rheum Dis. 2019 May;78(5):696-703

*Equal contribution

ASSOCIATED PAPERS

1. **Response to Letter to the Editor by Bartoloni et al: 'Interplay of anti-SSA/SSB status and hypertension in determining cardiovascular risk in primary Sjögren's syndrome'**
   Johannes Mofors, Marie Wahren-Herlenius, Gunnel Nordmark
   J Intern Med. 2020 Feb;287(2):216-217

2. **Effects of maternal medication on long-term outcome in congenital heart block remain to be established. Response to: 'Comorbidity and long-term outcome in patients with congenital heart block and their siblings exposed to Ro/SSA autoantibodies in utero' by Satis et al**
   Johannes Mofors, Sven-Erik Sonesson, Marie Wahren-Herlenius
   Ann Rheum Dis. 2020 Aug;79(8):e95

3. **Environmental factors in the pathogenesis of primary Sjögren's syndrome**
   Albin Björk, Johannes Mofors, Marie Wahren-Herlenius
   J Intern Med. 2020 May;287(5):475-492

4. **Viral antigens elicit augmented immune responses in primary Sjögren's syndrome.**
   Albin Björk, Gudny Ella Thorlacius, Johannes Mofors, Elina Richardsdotter Andersson, Margarita Ivanchenko, Joanna Tingström, Tojo James, Karl A Brokstad, Rebecca J Cox, Roland Jonsson, Marika Kvarnström, Marie Wahren-Herlenius
   Rheumatology (Oxford). 2020 Jul 1;59(7):1651-1661

5. **Sex differences in clinical presentation of systemic lupus erythematosus**
   Jorge I Ramírez Sepúlveda, Karin Bolin, Johannes Mofors, Dag Leonard, Elisabet Svenungsson, Andreas Jönsen, Christine Bengtsson, DISSECT consortium, Gunnel Nordmark, Solbritt Rantapää Dahlqvist, Anders A Bengtsson, Lars Rönnblom, Christopher Sjöwall, Iva Gunnarsson, Marie Wahren-Herlenius
   Biol Sex Differ. 2019 Dec 16;10(1):60

CONTENTS

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
AECG	American–European Consensus Group
AIDS	Acquired immunodeficiency syndrome
AIRE	Autoimmune regulator
ANA	Antinuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibody
AV	Atrioventricular
BAFF	B-cell activating factor
CD	Cluster of differentiation
CHB	Congenital heart block
CI	Confidence interval
DAG	Directed acyclic graph
DLBCL	Diffuse large B-cell lymphoma
DMARD	Disease-modifying anti-rheumatic drug
EIRA	Epidemiological Investigation of Rheumatoid Arthritis
ESSDAI	EULAR Sjögren's Syndrome Activity Index
ESSPRI	EULAR Sjögren's Syndrome Patient Reported Index
EULAR	European League Against Rheumatism
FDR	False discovery rate
GESS	Genes and Environment in Sjögren's Syndrome
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HR	Hazard ratio
ICD	International Classification of Diseases
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IQR	Interquartile range
IRF	Interferon regulatory factor
MALT	Mucosa-associated lymphoid tissue
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NF-κB	Nuclear factor-kappa B
NLE	Neonatal lupus erythematosus

NPR	National Patient Register
OR	Odds ratio
pSS	primary Sjögren's syndrome
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SLE	Systemic lupus erythematosus
SSA	Sjögren's syndrome antigen A
SSB	Sjögren's syndrome antigen B
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell

1 BACKGROUND

1.1 AUTOIMMUNE DISEASES

Autoimmune diseases arise from an abnormal immune response to an endogenous body part. More than 80 diseases with an autoimmune component have currently been described, collectively affecting approximately 4.5% of the world's population [1]. Autoimmune conditions can involve nearly all parts of the body, and often cause substantial increases in morbidity and mortality [2, 3]. The overall societal cost of autoimmune disorders is not known but is undoubtedly significant due to their chronic nature.

The treatment of autoimmune disorders generally aims to repress the erroneous immune response, thereby preventing or minimizing its harmful effects. Traditionally, drugs used to control disease activity include corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs), and other immunosuppressants. In recent years, protein-based drugs, targeting specific elements of the immune system, have be added to the therapeutic arsenal.

In spite of recent progress in understanding and treating autoimmune diseases, the mechanisms leading to their development are not fully elucidated. It is however clear that both genetic and environmental factors are central in the etiopathogenesis [4, 5]. An interplay between the two has repeatedly been proposed, where disease development is triggered by environmental factors in a genetically susceptible individual.

1.1.1 Genetic factors in autoimmune diseases

Autoimmune diseases have a definitive genetic component, which is evident from familial clustering of autoimmune diseases and from increased concordance rates in monozygotic twins compared to dizygotic twins or other siblings [6]. The median heritability of autoimmune disorders has been approximated to 60% [7], suggesting that genetic factors alone are pivotal, although not fully explaining autoimmunity.

Genome-wide association studies (GWAS) have identified a multitude of genetic variants throughout the genome associated with an increased or decreased risk of autoimmune diseases [8]. Although rare, there are known monogenic or Mendelian immune disorders that result in autoimmune diseases, often with a high penetrance and involving drastic immune phenotypes [9]. Generally, such disturbances in innate immunity, a system designed for universal sensing of danger signals, tend to result in systemic autoimmunity affecting multiple organs, whereas aberrations in adaptive immunity, which is built for antigen specificity, tend to result in conditions with organ-specific autoimmunity [10]. For example, C1q deficiency is a monogenic cause of autoimmunity which causes systemic lupus erythematosus (SLE) with a penetrance of nearly 100% [11, 12]. Meanwhile, mutations in the *Autoimmune Regulator* (*AIRE*) gene, which is central in promoting self-tolerance of T-cells via the expression of peripheral antigens in the thymus, is associated with organ-specific autoimmunity due to dysfunctional negative selection of self-reactive T-cells [13].

However, most genetic variants associated with autoimmune diseases only seem to convey a modest effect, implying a polygenetic setting. Moreover, multiple genetic variants

associated with autoimmunity are shared between several autoimmune diseases, suggesting the presence of common pathological pathways [14]. The most prominent such genetic variants are located within the human leukocyte antigen (HLA) region on chromosome 6, and explain a substantial part of known genetically attributed risk [8]. Polymorphisms in genetic regions affecting the expression of HLA genes have also been linked with autoimmune disorders [15]. Notably, extensive linkage disequilibrium among alleles and genetic variability has limited precise understanding of known associations.

Many loci not related to HLA expression have also been associated with autoimmune diseases, of which some are shared between different autoimmune conditions [8, 16]. Most of these genes in these loci belong to pathways important in immune responses, such as toll-like receptor (TLR) signaling and the type 1 interferon (IFN) system, B and T cell differentiation, immune cell activation and/or signaling, innate immunity, or tumor necrosis factor (TNF) signaling [17]. Specifically, genes implicated in multiple systemic autoimmune diseases include e.g*., STAT4, IRF5, PTPN22 and BLK* [18-21].

1.1.2 Environmental factors in autoimmune diseases

From relatively low concordance rates in monozygotic twins and estimates of heritability being far from 100%, it is apparent that genetic factors alone cannot explain the development of autoimmunity. However, compared to the amount of genetic research, the role of environmental risk factors has not been as thoroughly studied. Although substantial advances have been made in recent years, the identification of environmental risk factors is inherently problematic. Autoantibodies may develop many years before disease diagnosis, and it is now understood that autoimmune disorders generally develop progressively during long time intervals with initial nonspecific symptoms. Due to the vagueness and progressive onset, it is thus difficult to pinpoint possible triggers for disease development. Moreover, the identification of risk-factors in observational studies rely on high-quality exposure data and, ultimately, cannot establish causation. Nonetheless, epidemiological studies are crucial in the identification of potential environmental agents that may contribute to the breaking of immunological tolerance, allowing for subsequent assessment in experimental research.

For several autoimmune diseases, there is strong epidemiological evidence of associations with environmental factors, as well as interactions between specific genotypes and environmental agents [22, 23]. In an extensive review by Miller et al. [24], an expert panel assembled by the National Institute of Environmental Health Sciences assessed the accumulated evidence for the role of various environmental factors in the development of autoimmune disease. The panel divided environmental exposures into three broad classes: chemical agents, physical agents, and biologic agents.

Among chemical agents, exposure to crystalline silica has been extensively studied and may be considered an established risk factors for multiple autoimmune disorders including rheumatoid arthritis (RA) [25], systemic sclerosis [26], SLE [27], and anti-neutrophil cytoplasmic antibody (ANCA) related vasculitis [24]. Smoking has repeatedly been associated with an increased risk of autoantibody-positive RA; associations has also been suggested in other rheumatic diseases [24, 28]. Interestingly, gene-environment interactions have been demonstrated for smoking in RA [4] and multiple sclerosis (MS) [29], in which

smokers with certain risk genotypes have a considerably higher relative risk of disease. In contrast to other autoimmune diseases, findings have suggested that current-smoking may have a protective effect in ulcerative colitis [30]. Studies have also examined whether autoimmunity may arise from exposure to solvents. A robust relationship has been established in systemic sclerosis [31], and a similar pattern has been indicated in MS [32].

Physical factors, mainly in the form of radiation, have also been implicated in the onset of autoimmune diseases. Substantial data have shown an increased risk of autoimmune thyroiditis and Grave's disease after radiation treatment in cancer patients [33, 34]. Moreover, an inverse association exists between higher ultraviolet radiation exposure and lower risk of development of MS [35, 36]. Conversely, sunlight exposure is known to induce flares in SLE, although its role in the etiopathogenesis is unclear [24, 37, 38]. Related to sun exposure, low vitamin D levels have been linked to an increased risk of MS [23].

Biologic agents, mainly in the form of infections, have long been proposed as risk factors for development of autoimmune diseases. Various pathogens, including bacteria, viruses, and parasites, have been suggested as triggers. Such hypotheses often stem from the observation that a higher proportion of patients with autoimmune disease show seropositivity for certain microbes compared to controls, or that infected individuals may present with symptoms similar to those of patients with some autoimmune diseases [39]. Different mechanisms by which infections may induce autoimmunity have been proposed, including both innate and adaptive immunity originating from cross-reactivity between infectious agents and endogenous tissue [40]. Intriguingly, Epstein-Barr virus infections have been implicated in many autoimmune diseases such as RA [41], SLE [42, 43], MS [23], and Sjögren's syndrome [44]. Additionally, disturbances of gut mucosal tissue, microbiota, and immune homeostasis has been suggested to be of importance in the development of autoimmunity [45].

1.2 SJÖGREN'S SYNDROME

Primary Sjögren's syndrome is a systemic rheumatic disease characterized by an immune-mediated exocrinopathy, resulting in dryness of the eyes and mouth. The clinical presentation often includes fatigue and joint pain, and a subset of patients develop a more severe condition with multi-organ involvement [46, 47]. The disease has a major adverse impact on the quality of life, primarily because of fatigue and loss of work productivity [48]. Immunological features of primary Sjögren's syndrome include abnormalities of both the innate and adaptive arm of the immune system. There occurs formation of ectopic lymphoid tissue in the salivary glands, activation of the type I IFN system, and production of autoantibodies to the Sjögren's syndrome antigen A (Ro/SSA) and Sjögren's syndrome antigen B (La/SSB) in a subset of patients [46].

The condition may occur in isolation, in which case the disease is referred to as primary Sjögren's syndrome. It may also arise with another systemic autoimmune disease, such as RA, SLE, systemic sclerosis, or dermatomyositis, in which the condition is referred to as an overlap syndrome or secondary Sjögren's syndrome.

1.2.1 Epidemiology

The disease has a female-to-male predominance of 9-14:1 [47, 49]. The peak incidence is at approximately 50 years of age for females, and more evenly distributed in the male population [49]. A limited number of epidemiological studies are published, with results varying widely depending on method used for estimation, ethnicity of investigated cohorts, and classification-criteria used. A recent study in Sweden estimated incidence rate of the disease to 3.9 per 100,000 person-years [50], whereas a meta-analysis estimated a pooled incidence rate of 6.9 [49]. The prevalence of primary Sjögren's syndrome is estimated to 0.01 to 0.6% of the population [49, 50].

1.2.2 Diagnosis

No single sign, symptom or diagnostic test defines Sjögren's syndrome. Instead, the diagnosis is based on the joint presence of subjective symptoms and objective signs of impaired ocular and oral glandular function, presence of disease specific autoantibodies (Ro/SSA and La/SSB), and histological examination of minor salivary gland biopsies. Different classification criteria are available to aid the diagnosis, although these are not diagnostic, but developed for the purpose of creating well-defined patient groups for research purposes [47, 51].

Many national classification criteria have been developed and used locally, but an international effort in 2002 established the so-called American-European consensus group (AECG) criteria that can be used in classifying primary Sjögren's syndrome [51] (**table 1**). These criteria have been widely adopted clinically and used for research purposes. The criteria include subjective and objective measures of ocular and oral exocrine dysfunction, serological presence of autoantibodies to the Ro/SSA and/or La/SSB antigen, and a minor salivary gland biopsy showing focal lymphocytic sialoadenitis. One of the two latter must be fulfilled for a diagnosis of primary Sjögren's syndrome in accordance with the AECG criteria.

I	**Subjective ocular symptoms** positive response to one or more of the following claims: *1) daily persistent trouble with dry eyes for more than 3 months* *2) recurrent sensation of sand or gravel in the eye* *3) usage of tear substitutes more than three times per day*
II	**Subjective oral symptoms** positive response to one or more of the following claims: *1) daily feeling of dry mouth for more than three months* *2) recurrent or persistent swollen salivary glands as an adult* *3) need frequently drink liquids to aid swallowing dry food*
III	**Objective ocular involvement** evidence of ocular involvement, determined on the basis of positive result in at least one of the following tests: *1) Schirmer's test (positive if ≤ 5mm/5min)* *2) Van Bijsterveld score (positive if ≥ 4 points on a 0-9-point scale)*
IV	**Histopathology of minor salivary gland** focus score of ≥ 1 in a minor salivary gland biopsy (focus score defined as the number of foci per 4 mm^2 of glandular tissue; focus defined as a conglomerate of more than 50 lymphocytes)
V	**Objective salivary gland involvement** unstimulated whole salivary flow (≤1.5 mL in 15 minutes)
VI	**Serology** presence of Ro/SSA and/or La/SSB autoantibodies in serum

Table 1. **The AECC criteria for classification of primary Sjögren's syndrome, adapted from Vital et al. [51].**
Any four of the six items should be fulfilled, provided that IV or VI is positive.
Alternatively: presence of three out of the four objective criteria (III–VI).
Exclusion criteria: Past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency disease (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease, use of anticholinergic drugs.

A new set of classification criteria were presented by the American College of Rheumatology (ACR) in 2012 [52], but never reached wide usage. The unsuccessful transition was attributed to the older criteria working well, the new criteria lacking robust validation, and the requirement for ocular staining by a trained ophthalmologist. More recently, the European League Against Rheumatism (EULAR) and the ACR together developed and approved the new 2016 ACR/EULAR criteria [53] (**table 2**). The new diagnostic criteria are similar to older AECG criteria, which is evident from a high concordance rate [53, 54]. However, in contrast to the AECG criteria, the ACR/EULAR does not consider the presence of La/SSB autoantibodies, pre-existing lymphoma is not an exclusion criterion, and immunoglobulin G4 (IgG4)-related disease (recognised as a disease entity in 2012) is an exclusion criterion.

Item	Score
Labial salivary gland histology with a focus score ≥1 (defined as a number of lymphocytic foci per 4 mm^2, where a focus is defined as a conglomerate of >50 lymphocytes)	3
Presence of Ro/SSA autoantibodies in serum	3
Ocular staining score ≥5 (or van Bijsterveld score ≥ 4) in at least one eye	1
Schirmer's test ≤5 mm/5 min in at least one eye	1
Unstimulated whole saliva flow rate ≤ 0.1 ml/min	1

Table 2. **The ACR/EULAR criteria for classification of Sjögren's syndrome, adapted from Shiboski et al. [53].**
The classification of primary Sjögren's syndrome applies to individual who meets the inclusion criteria,* does not have any exclusion criteria,† and has a total score of ≥4.

*Defined as a positive response to ≥1 of the following questions:
(1) Have you had daily, persistent, troublesome dry eyes for more than 3 months?
(2) Do you have a recurrent sensation of sand or gravel in the eyes?
(3) Do you use tear substitutes more than three times a day?
(4) Have you had a daily feeling of dry mouth for more than 3 months?
(5) Do you frequently drink liquids to aid in swallowing dry food?
Or in whom there is suspicion of Sjögren's syndrome from the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index questionnaire (at least one domain with a positive item).

† Defined as individuals having any of the following conditions:
(1) history of head and neck radiation treatment
(2) active hepatitis C infection
(3) AIDS
(4) sarcoidosis
(5) amyloidosis
(6) graft-versus-host disease
(7) IgG4-related disease

1.2.2.1 Disease activity monitoring

In order to aid a systematic and uniform measurement of disease activity and burden of symptoms, two indices have been developed: the EULAR Sjögren's Syndrome Activity Index (ESSDAI) [55] and the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) [56]. In the ESSDAI index, disease activity across twelve weighted domains for different organ systems is categorized from absent to high by a clinician. A total score is calculated by adding the individual domains scores, giving a total score from 0 to 123. The index is developed to identify active and thus potentially treatable inflammation. The ESSPRI score is obtained by calculating the mean of patient-reported symptoms for dryness, pain and fatigue, each graded from a scale of 0 to 10. Reports on the correlation between the two scores have varied [57, 58], underlining that the scores should be considered as complementary.

1.2.3 Etiology and pathogenesis

The etiopathogenesis of Sjögren's syndrome is not well understood, but disease development is believed to result from environmental factors triggering a genetically predisposed individual [59]. Several specific genetic polymorphisms have been associated with Sjögren's syndrome, with the prominent association found in the region of major histocompatibility complex (MHC) [60, 61]. Notably, the association with the MHC locus is more strongly related to the production of Ro/SSA and La/SSB autoantibodies than the diagnosis itself [62]. Other genetic loci with genome-wide significant association of Sjögren's syndrome with polymorphisms are *STAT4, IL12A, TNIP1, IRF5, BLK-FAM167A,* and *CXCR5* in Caucasians [63]. The indicated genes can be assigned to the NF-κB pathway, IFN signaling, lymphocyte signaling, and antigen presentation [19].

By contrast, little is known about environmental risk factors in primary Sjögren's syndrome. A link between infections and Sjögren's syndrome has repeatedly been proposed, with both viruses and bacteria suggested as triggers for disease development [64-66]. Such hypotheses mainly stem from observations where higher proportions of Sjögren's syndrome patients showed seropositivity to specific pathogens compared to controls [66], or that individuals infected with some pathogens may present symptoms similar to those of patients with Sjögren's syndrome. However, a recent study demonstrated how bacteria from skin and mucosal tissue containing orthologs of the Ro60 protein activated antigen-specific $CD4^+$ memory T cells from SLE patients, as well as inducing Ro/SSA autoantibodies and signs of autoimmunity in a mouse model, thus highlighting the feasibility of infections as triggering agents [67]. Recently, in a review paper by Björk et al. [68], an overview of how inflammation secondary to infections may trigger autoimmunity was presented (**figure 1**).

A wide array of other environmental risk factors have been implicated in the development of the disease, such as exposure to smoking, solvents, silica, low levels of vitamin D, and hormone levels. However, findings are generally inconsistent and with no definitive conclusions [68].

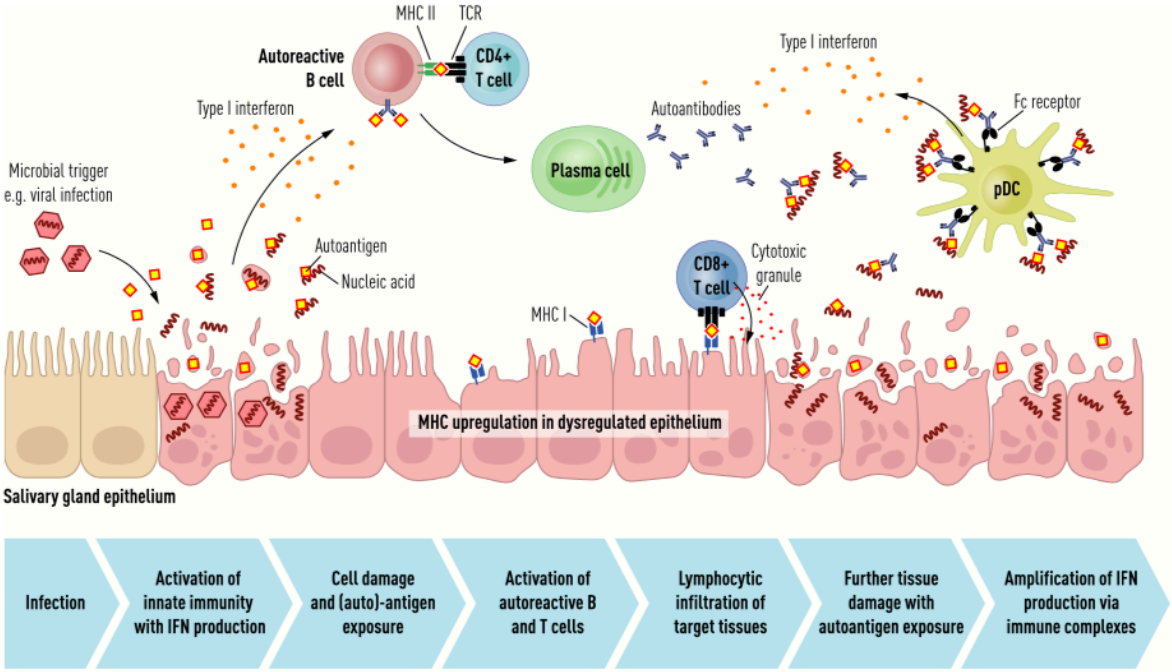

Figure 1. Potential immunopathogenic mechanism in the development of Sjögren's syndrome. Infectious agents induce inflammation in the salivary gland epithelium with the production of type I IFN and upregulation of MHC molecules. Exogenous and endogenous antigens are presented to B and T cells, which lead to the activation and proliferation of autoreactive clones. Autoreactive cytotoxic T cells induce further tissue damage, thus amplifying the exposure of autoantigens. Autoantibodies produced from B cells bound to autoantigens form complexes with plasmacytoid dendritic cells, resulting in augmented type I IFN production. Thus, a feed-forward loop of autoimmunity is created. IFN, interferon; MHC, major histacompatability complex; pDC, plasmacytoid dendritic cells; TCR, T cell receptor. Figure reprinted with permission from the publisher [68].

1.2.3.1 Pathogenesis

The current model for the immunopathogenesis in Sjögren's syndrome is a self-sustaining cycle of immune activation. Alterations in the type I IFN system and B cell-function are central elements, although T-cells and innate immune cells also are implicated in the disease [46, 69].

Briefly, a triggering factor (possibly an infection) leads to the production of type I IFN in mucosal epithelial cells, which results in the recruitment and activation of immune cells. Type I IFN signaling leads to a subsequent activation of dendritic cells which process and present self-antigens from dying cells to autoreactive T cells. Cytotoxic T cells kill healthy cells expressing high levels of antigen, whereas T helper (Th) cells and type I IFN promotes the differentiation of autoreactive B cells to plasma cells secreting antibodies (such as Ro/SSA and La/SSB autoantibodies). Autoantibodies bound to self-antigens consisting of nucleic acid binding proteins, such as Ro/SSA and La/SSB, in cell debris, bind to Fc receptors on plasmacytoid dendritic cells, which via TLR 7 and 9 signaling propagate the response through the production of large quantities of type I IFN [46, 70, 71].

Patients with primary Sjögren's syndrome also have increased serum levels of B-cell activating factor (BAFF), induced by type I and type II IFN. It is another cytokine that is believed to be central in the immunopathology through its ability to promote B-cell proliferation and differentiation into antibody-producing plasma cells [46, 69]. The Ro/SSA and La/SSB antibodies are present in about 70–75% and 40–45% of the patients,

respectively, and associate strongly with disease severity and extraglandular manifestations of the disease [50, 69, 72, 73].

In addition, the formation of ectopic lymphoid structures in glandular tissue, so-called germinal centers, are considered central in establishing the chronic inflammation in Sjögren's syndrome. Indeed, the grade of lymphocytic infiltration and formation of germinal centers have been correlated to disease severity and systemic complications [74].

1.2.3.2 Ro/SSA and La/SSB antigens

The Ro/SSA antigen consists of the Ro52 and Ro60 proteins, located intracellularly in the cytoplasm or nucleus. The proteins were previously thought to be part of the same complex, but are now suggested to be separate structures with no known physiological association to each other.

The expression Ro52 is induced from viral infections and TLR-signalling, and has anti-inflammatory effects in its role as an E3 ligase in the ubiquitination process. It has been shown to downregulate IRF3, IRF7 and NF-κB signalling [75-77]. Meanwhile, the function of Ro60 is not known, but has been shown to bind to non-coding RNA. It has been suggested to target misfolded RNA for degradation, as well as binding to viral RNA. Interestingly, antibodies against EBV has been shown to cross-react with Ro60 [78], supporting the theory of EBV having a triggering role in the development of Ro/SSA antibodies.

The La/SSB is a protein involved in the metabolism of non-coding microRNAs, and is located between the nucleus and cytoplasm [79]. The production of anti-La/SSB autoantibodies is generally thought to arise from epitope spreading from antibodies targeting Ro/SSA. This concept has been demonstrated in mouse models [80], and is evident from patients with La/SSB autoantibodies with few exceptions also having antibodies targeting Ro/SSA [73].

Ro/SSA antibodies are often present in other autoimmune diseases such as SLE, RA, systemic sclerosis, primary biliary cirrhosis, and myositis [81]. In contrast, La/SSB antibodies are more closely associated to Sjögren's syndrome and SLE [82]. Other autoantibodies frequently present in Sjögren's syndrome are anti-nuclear antibodies (ANA) and rheumatoid factor (RF), present in about 80% and 50% of patients, respectively [73].

1.2.4 General symptoms and extraglandular manifestations

Most patients with Sjögren's syndrome have general symptoms in common with many other systemic inflammatory diseases, such as fatigue and muscle pain, resulting in increased rate of work disability and reduction of quality of life [47, 48, 83]. Systemic complications occur in around 30 and 40% of the patients, and are often referred to as extraglandular manifestations. They might develop either at the onset of the disease or later. The complications can be widespread, involving most organ systems of the body [47, 72, 84].

Some of the more frequent symptoms include arthralgia, skin involvement such as cutaneous vasculitis and purpura, and pulmonary conditions with bronchial or parenchymal

involvement [85]. A subset of patients also has renal complications, mainly in the form of interstitial nephritis or cryoglobulinemia associated glomerulonephritis [86]. Sjögren's syndrome can also cause peripheral neuropathy and to a lesser extent, inflammation in the central nervous system such as myelitis and cerebral lesions [87]. Hematological and serological aberrations may also be observed in patients, including anemia, thrombocytopenia, neutro- or lymphopenia, hypergammaglobulinemia, and hypocomplementemia [47, 72, 84].

1.2.4.1 Lymphoproliferative disease

Patients with primary Sjögren's syndrome have an estimated 15 to 20 times higher risk of lymphoma compared to the general population, making it the highest risk among rheumatic diseases and corresponding to a lifetime risk of 5 to 10% [88, 89]. The lymphomas are mostly B-cell non-Hodgkin's lymphomas, with a predominance of the low-grade type [90-94].

The risk of lymphoma development in Sjögren's syndrome is reported to increase with disease duration and is associated with various clinical risk factors. Significant predictors for the development include low complement levels, presence of cryoglobulins, low CD4/CD8 ratio, recurrent parotid gland swelling, lymphadenopathy, splenomegaly, and palpable purpura [91, 95-97]. Newer markers for the risk of lymphoma in Sjögren's syndrome have also been suggested, including elevated serum BAFF levels [98], elevated levels of Flt3-ligand [99], and genetic impairment of *TNFAIP3* [100]. Despite the current knowledge of risk factors for lymphoma development, there is no consensus regarding the monitoring of Sjögren's syndrome patients having one or multiple risk factors.

The mechanisms driving the increased risk of lymphoproliferative disease in Sjögren's syndrome is not fully elucidated, but has been attributed to the chronic B-cell activation in this condition. Moreover, dysregulation of Th17 and regulatory T cells (Tregs) cells have also been implicated in the pathogenesis [101]. Further, acquired mutations in the TNFAIP3 protein, central in regulating NF-κB activation, has been observed in Sjögren's syndrome associated lymphoma [102]. Also, signaling abnormalities in the NOTCH pathway, which too is implicated in autoimmune diseases, have been suggested to contribute to lymphoma development [103].

Extranodal mucosa-associated lymphoid tissue lymphoma (MALT), originating from B-cells, is the most frequent lymphoma subtype in Sjögren's syndrome patients [90-94]. They are typically localized in the salivary glands, but may also present in other mucosal tissues such as lacrimal glands, nasopharynx, stomach, and lungs. These lymphomas are generally indolent and with a good prognosis. Meanwhile, the incidence of high-malignant diffuse large B-cell lymphoma (DLBCL) is also increased in Sjögren's syndrome patients, and have in some cases been reported to evolve from previous low-grade lymphomas [94, 104, 105]. The occurrence of other malignancies evolving from mature B-cells, such as Hodgkin's lymphoma and multiple myeloma have not been thoroughly studied in Sjögren's syndrome, although studies have indicated that there might be an increased risk [106-108]. Moreover, an increased prevalence of monoclonal gammopathy of unknown significance (MGUS) in Sjögren's syndrome, associated with an increased risk of multiple myeloma [109], has been reported [110, 111].

1.2.5 Treatment

Treatment of primary Sjögren's syndrome is generally based on the degree of symptoms and systemic manifestations. Topical symptomatic treatment is usually considered appropriate, whereas systemic treatment is used in patients with extraglandular manifestations.

Oral and ocular symptoms are managed by tear and saliva substitutes, education, environment modification, and elimination of contingent offending drugs [112]. Moreover, randomized clinical trials have established the benefit of muscarinic agonists for the treatment of oral and ocular dryness in Sjögren's syndrome [113, 114].

So far, no immunomodulatory drug has been proven effective for the systemic treatment of Sjögren's syndrome in clinical trials. Disease heterogeneity is one major factor complicating the evaluation of examined agents. Currently, severe manifestations are treated in accordance with guidelines for SLE or other connective-tissue diseases [47, 112].

Milder symptoms of pain and arthralgia in Sjögren's syndrome are often treated with analgesics, such as non-steroidal anti-inflammatory drugs. Moderate symptoms such as cutaneous manifestations and arthritis are widely treated with hydroxychloroquine, sometimes in combination with low-dose corticosteroids. Hydroxychloroquine interferes with the signaling of TLR in endosomes, thereby inhibiting the IFN production [115]. Various smaller studies have reported beneficial effects on symptoms and extraglandular manifestations from hydroxychloroquine in Sjögren's syndrome, yet with negative results in larger trials [116-118]. Immunosuppressive drugs for severe systemic manifestations include methotrexate, azathioprine, and cyclophosphamide [47, 112].

More recently, the use of several biological agents has been investigated in Sjögren's syndrome. There are some reports on the beneficial effects from B-cell depletion using anti-CD20 antibodies (Rituximab), as measured by tear and saliva production and subjective measures [119-121], although findings from larger studies have been insufficient [122, 123]. Inhibition of BAFF signaling using the monoclonal antibodies Ianalumab and Belimumab has also been investigated, with ambiguous results with regard to improvement of disease activity, and with no persistent improvement of exocrine function [124-126]. Inhibition of T-cell activation using a CTLA-4 fusion protein (Abatacept) has also been investigated. While initial smaller investigations reported improvements in ESSDAI and ESSPRI scores, as well as improved salivary flow, beneficial effects could not be established in a recent phase 3 trial [127-129]. Several other efforts targeting IFN signaling and co-stimulatory pathways are also ongoing, and may provide an effective way to control the disease [112, 130].

1.3 CARDIOVASCULAR DISEASE AND INFLAMMATION

1.3.1 Atherosclerosis

Cardiovascular disease is one of the leading causes of mortality globally, with coronary artery disease and cerebrovascular disease being the most frequent [131]. Their underlying pathological process is atherosclerosis, a slowly progressive disorder of large and medium-sized arteries that becomes clinically overt when it causes tissue hypoxia and/or thrombosis [132].

Atherosclerosis was long considered to be a passive accumulation of cholesterol in the arterial vessel wall. Today, innate and adaptive immune responses are recognized in the pathogenesis, where components of cholesterol-carrying lipoprotein triggers inflammation, T cell activation and antibody production [133]. In recent years, it has been demonstrated that systemic inflammation can enhance atherogenesis [134]. Indeed, increased morbidity and mortality related to cardiovascular disease has been reported for patients with systemic rheumatic diseases, such as RA and SLE [135-137].

1.3.2 Venous thromboembolism

Venous thromboembolism, with a reported incidence of 1 to 2 cases per 1,000 person-years, poses a major medical problem with a substantial 30-day mortality [138, 139]. Numerous medical conditions, such as hospitalization, surgery, malignancy, immobilization, and thrombophilic states are well recognized risk factors [138].

Moreover, venous thromboembolism has also been linked to inflammation. Several immune-mediated disorders, such as RA and SLE have been increasingly recognized as predisposing factors, and it is suggested that hypercoagulability is a general feature of most systemic autoimmune diseases [140, 141]. The underlying mechanisms for this association are not completely known, but have been suggested to relate to vasculopathy and chronic inflammation increasing coagulability [142, 143]. Inflammation may increase coagulability mainly by cytokine induced tissue-factor expression, downregulation of the protein C system and the inhibition of fibrinolysis [142].

1.3.3 Cardiovascular disease in primary Sjögren's syndrome

Primary Sjögren's syndrome shares many clinical, inflammatory and immunological features with RA and SLE. However, primary Sjögren's syndrome frequently presents with a more benign course, often without the need for immunosuppressive therapy [47]. Previous studies have suggested that, compared with the general population, patients with primary Sjögren's syndrome have a higher frequency of traditional risk factors associated with cardiovascular disease, including hypertension, hypercholesterolemia, and hypertriglyceridemia [144-146]. The prevalence of diabetes has, however, shown conflicting results [72, 144, 145]. Moreover, subclinical atherosclerosis and endothelial dysfunction has been described in different settings, reviewed by Valim et al. [147].

Several population-based studies have investigated the association between primary Sjögren's syndrome and cardiovascular disease, including myocardial infarction, cerebral infarction, and venous thromboembolism [141, 144, 145, 148-152]. Although somewhat

inconsistent, the results indicate an increased incidence of clinically manifest cardiovascular disease. However, the studies suffer from not including patients verified by established diagnostic criteria, or being able to stratify them based on the presence of Ro/SSA and La/SSB autoantibodies. The presence or absence of these autoantibodies mark two clinically and genetically distinct populations [62, 69, 72], and are thus relevant to account for when assessing comorbidity.

1.3.4 Antibody-mediated congenital heart block

Complete congenital heart block (CHB) without cardiac malformation is a rare condition, affecting approximately 1 in 23,000 births in the general population [153]. The development of CHB may be caused in fetuses exposed to maternal Ro/SSA and La/SSB autoantibodies [154-156], and occurs in 1 to 2 percent of exposed fetuses [156-158]. Women carrying the autoantibodies are often diagnosed with SLE or Sjögren's syndrome, but may also be asymptomatic [159-161].

The maternal autoantibodies are transported across the placenta during pregnancy, and may induce neonatal lupus (NLE) in the fetus, including a complete third degree atrio-ventricular (AV) block [162-164]. The conduction dysfunction of the disorder is associated with decreased cardiac function, and the majority of children with CHB require a pacemaker at an early age [165].

Given the rarity of the condition, few studies have systematically assessed comorbidity and complications in later life in patients with CHB. There have however been reports on growth restrictions during early life [166, 167], as well as an increased prevalence of impaired neurodevelopment [167], and neuro-psychiatric abnormalities [168]. Furthermore, recent case reports have also suggested CHB to be a risk factor for the subsequent development of autoimmune disorders [169-174]. Whether siblings to CHB patients unaffected by the cardiac malformation share this risk is not clear [175], although familial aggregation of autoimmune disorders in general suggests that it might be the case [176, 177].

2 AIMS OF THE THESIS

The objectives of the work performed in this thesis was to study potential triggers that contribute to disease development of primary Sjögren's syndrome, and to investigate comorbidity associating with the disease.

The specific aims of the thesis were to:

I. Investigate infections as a potential trigger of primary Sjögren's syndrome.

II. Investigate the association between cigarette smoking and subsequent development of primary Sjögren's syndrome.

III. Study the risk of incident cardiovascular disease in individuals with primary Sjögren's syndrome.

IV. Investigate the risk of multiple myeloma in patients with primary Sjögren's syndrome.

V. Study comorbidity in individuals born to anti-Ro/SSA-positive mothers with congenital heart block.

3 MATERIAL AND METHODS

3.1 STUDY POPULATIONS

3.1.1 Individuals with primary Sjögren's syndrome

A study population of patients with primary Sjögren's syndrome, used in **Study I–IV**, was established through a joint effort from the Departments of Rheumatology at the University Hospitals in Gothenburg, Linköping, Malmö/Lund, Uppsala, Örebro, and the Karolinska University Hospital in Stockholm, in which patients fulfilling the AECG criteria [51] were compiled.

In total, n=1,009 patients diagnosed with Sjögren's syndrome between 1967 and 2013 were identified. The average age at diagnosis was 56 years, 93% of the cases were female. Two thirds were Ro/SSA and/or La/SSB antibody-positive, thus reflecting the composition of the disease population as described in the literature (**table 3**).

Table 3. Demographic and clinical variables of included patients with primary Sjögren's syndrome.

Diagnosing clinic	No. individuals	Females	Median age at diagnosis (IQR)	Ro/SSA and/or La/SSB positive	Ro/SSA positive	La/SSB positive
Göteborg	56	96%	56 (45–63)	71%	71%	36%
Linköping	238	93%	60 (47–68)	79%	79%	42%
Malmö	405	92%	55 (43–65)	66%	64%	48%
Stockholm	171	94%	55 (45–65)	58%	53%	37%
Uppsala	118	91%	53 (42–62)	75%	75%	38%
Örebro	21	81%	56 (51–60)	81%	81%	43%
Total	**1,009**	**93%**	**56 (45–66)**	**69%**	**68%**	**43%**

Abbreviations: IQR, interquartile range.

A control group was established by randomly selecting ten individuals from the Swedish Population Register (which includes all residents in Sweden) for each unique patient with Sjögren's syndrome, matched for sex, year of birth, and residential area ten years before the date of Sjögren's syndrome diagnosis (n=10,090). Each control individual was assigned the same index date as the corresponding patient with Sjögren's syndrome.

Subgroups from the above patients and controls were used in **Study I–IV**. The subgroups of individuals included in **Study I**, **III**, and **IV** was determined by index dates being required to occur within the observational periods as determined by registers used to identify morbidity (see below). In addition, controls were required to be alive at the last day of the observational periods in **Study I**, as well as not have emigrated at start of follow-up in **Study III** and **IV**.

The individuals with Sjögren's syndrome who in 2017 were alive and had not emigrated (n=815), were invited to participate in a questionnaire of lifestyle habits. The answers from those returning the questionnaire (n=606), was used in **Study II**.

3.1.2 Individuals with congenital heart block

Individuals with CHB without cardiac malformation were identified in 2011 using the Pacemaker Register [178], and local university hospital registers [179]. The CHB condition was required to be detected prior the age of 15 years, and mothers required to have Ro/SSA and/or La/SSB autoantibodies.

The CHB population, used in **Study V,** consisted of n=119 individuals, born between year 1948 and 2010. Siblings of the CHB patients (n=128), defined as individuals sharing both parents, were also included in the study. For each patient with CHB, ten controls from the general population (n=1,190; matched for sex, year and month of birth, and region of birth) were randomly selected from the Swedish Total Population Register. Siblings of the controls were also identified (n=1,071), and were used as controls for the siblings of the individuals with CHB.

3.2 DATA SOURCES

3.2.1 Registers to identify morbidity

The occurrence of disease in **Study I**, **III**, and **V** was identified using the National Patient Register (NPR). The register has a nationwide coverage of diagnoses from hospitalizations since 1987 and for outpatient care (excluding diagnoses from primary care) since 2001. The NPR records data on the date of admission, date of discharge and discharge diagnoses as set by the discharging physician. The diagnoses are classified according to the calendar year-specific version of the International Classification of Diseases (ICD). The coverage is 99% for hospitalizations and 80% for outpatient care, the latter's lower coverage being mainly due to lower reporting from private and psychiatric health care providers [180]. Although depending on the type of diagnosis, the register has been externally confirmed to maintain a high validity [180]. In addition to the NPR, the Cause of Death Register was used to identify records of cardiovascular morbidity in **Study III**. The register comprises data on all deaths of people registered in Sweden since 1952.

The risk of hematological malignancy in Sjögren's syndrome was studied in **Study IV**, for which the Swedish Cancer Register was used. This register also classifies diagnoses according to ICD, and has a nationwide coverage from 1958 and onwards.

Through linkage with the above registers, data on morbidity for the cohort of Sjögren's syndrome patients and controls was available until death, emigration, or the 31st of December 2013, whichever came first. Equivalently, data from the above registers was linked and extracted until 31st of December 2010 for the individuals with CHB, their siblings, and their respective controls.

3.2.2 Data on smoking exposure

To investigate the association between cigarette smoking and Sjögren's syndrome in **Study II**, data on self-reported smoking habits in cases with Sjögren's syndrome and control individuals was used.

Individuals from the above Sjögren's syndrome population who in 2017 were alive and had not emigrated (n=815) were invited to participate in a questionnaire study called the Genes and Environment in Sjögren's Syndrome (GESS) study. The questionnaire consisted of 92 questions on various demographic and life-style aspects, such as education, work, dietary and smoking habits. N=606 individuals returned the questionnaire, corresponding to a response-rate of 74%.

For comparison, data from the equivalent questions were used from the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) I [181] and EIRA II [182] studies, which are part of a population-based project comprising the Swedish population, performed during 1996 to 2014. For each patient with Sjögren's syndrome, up to fifteen unique responders from the EIRA study were randomly selected based on sex, date of birth, area of residency, and calendar time at index date. The index date was defined as the date of Sjögren's syndrome diagnosis for cases, and the date of responding to the questionnaire for controls. Ultimately, n=530 patients with Sjögren's syndrome responding to the GESS questionnaire were matched to n=4,425 individuals from the EIRA studies.

In addition, data on HLA available for 68% and 47% of the cases and controls, respectively, was included in some analyses. In analyses stratified by specific HLA haplotypes, controls were additionally required to have at least one allele (i.e. heterozygotic) of the haplotype of interest.

3.3 STUDY DESIGNS

Study I was a case-control study, where exposure to infections preceding the diagnosis of Sjögren's syndrome was assessed and compared to controls. Data from the inpatient and outpatient register in NPR was used to identify infections, which were classified as either any infection, respiratory infections, skin infections, urogenital infections, and infections in the gastrointestinal tract. To mitigate distorting effects from reversed causality, a latency period of one year was applied, where preceding infections occurring within one year's proximity to the index date were not considered as exposures.

Study II was a questionnaire-based case-control study, in which exposure to cigarette smoking prior to Sjögren's syndrome was analyzed using data from the GESS and EIRA questionnaires. Only regular smoking was considered as exposure. In addition to dichotomously categorizing individuals as ever-smokers or non-smokers, longitudinal patterns of smoking and the quantity of smoking exposure was assessed.

Study III was performed as a cohort study, were the risk of incident cardiovascular disease defined as myocardial infarction, cerebral infarction, and venous thromboembolism was investigated in individuals with Sjögren's syndrome and matched controls. Data on cardiovascular morbidity was obtained from the NPR.

Study IV was also a cohort study, consisting of individuals with Sjögren's syndrome and matched controls. The incidence and risk of multiple myeloma was analyzed using data from the Swedish Cancer Register.

Study V was a cohort study comprising of individuals with CHB, their siblings, and matched controls. The risk of morbidity from start of follow-up, defined as either 1987 or date of birth if occurring after 1987, was assessed with an exploratory approach using data from NPR.

3.4 METHODOLOGICAL CONSIDERATIONS

3.4.1 Sources of bias

Systematic error, commonly referred to as bias, may arise in observational studies and distort estimations of association between variables. Bias can cause both under and over-estimation of the effect of an exposure on an outcome. Thus, adequate identification of sources of bias is central when trying to mitigate its skewing effects, as well as when assessing observed relationships.

Directed acyclical graphs (DAGs) may be used to identify sources of bias (**figure 2**). A DAG displays the assumptions about the relationship between variables, where the relationship between variables (or nodes) are denoted by edges. As the name implies, DAGs are finite directed graphs without implied cycles.

Figure 2. A simple DAG where an exposure (E) causes an outcome (O).

3.4.1.1 Confounding

A confounder is a variable that that exerts effect both on the exposure (independent) and outcome (dependent) variable. In the presence of a confounder, its effect on the outcome variable must be accounted for in order to obtain an unbiased estimate of the effect from the exposure variable (e.g. by inclusion as an independent variable, or by matching on the confounding variable). Theoretically, a studied exposure variable may have no causal effect on an outcome variable – upon which statistical inference between the exposure variable and an outcome variable would only (spuriously) capture the effect from the confounder. A generic example in which an exposure variable is confounded is illustrated below (**figure 3)**.

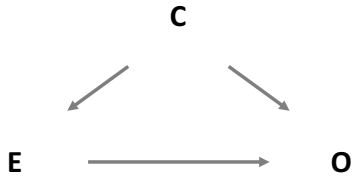

Figure 3. A DAG where a confounding variable (C) affects both exposure (E) and outcome (O) variable.

Relatedly, an exposure variable may exert its effect on the outcome directly and/or indirectly via a mediator variable (**figure 4**). As opposed to confounding variables, not accounting for the effect of the mediator variable does not inherently imply bias when quantifying the effect from the exposure variable on the outcome.

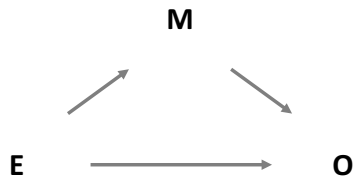

Figure 4. A DAG where an exposure (E) directly causes and outcome (O), and indirectly via a mediator (M).

3.4.1.2 Selection bias

The selection of individuals included in a study can potentially adversely affect the ability to make unbiased estimates of the relationship between an exposure and an outcome. More specifically, *selection bias* occurs when inclusion in a study depends on both exposure and outcome, leading to biased estimates of the relationship between exposure and outcome (internal validity). Conversely, estimates of association will not inherently be biased if the likelihood of participation in a study depend on exposure status only (or outcome status only).

Relatedly, a skewed sampling of individuals from a population, causing some members of the population to be less or more likely to be included than others (e.g. with specific characteristics), may not necessarily result in selection bias. However, the ability to make generalizable findings (external validity) will likely be affected.

3.4.1.3 Measurement bias

The correct measurement and classification of individuals with regard to exposure and outcome status will also affect the ability to produce undistorted estimates. The incorrect classification of individuals is called *misclassification*, and may lead to bias. A central concept is whether the misclassification is differential or non-differential. *Differential misclassification* exists when measurement of exposure status is affected by outcome status (or the other way around), whereas the measurement error is independent in *non-differential misclassification*. Differential misclassification error can either increase or decrease estimated associations, whereas non-differential misclassification normally affects estimates towards the null (i.e. dilutes associations).

Differential misclassification of the exposure may arise in questionnaire-based case-control studies, such as **Study II**. This is often referred to as *recall bias*, and exists when the self-reported exposure status is influenced by the outcome status (case or control).

3.5 STATISTICAL ANALYSES

3.5.1 Logistic regression

Logistic regression is a statistical model that can be used to model a binary dependent variable, such as diseased/not diseased. The predictor variable(s), may however be either continuous or categorical.

It is a so-called generalized linear model that models the log-odds of the dependent variable (the natural logarithm of the odds of it occurring) given the independent variable(s) by applying maximum likelihood estimation. Thus, the logistic regression estimates the odds of a certain event occurring or not. In a univariate setting, the model can be expressed as:

$$\log(odds\ of\ event\ occuring\ |X) = \beta_0 + \beta_1 X$$

Two main logistic regressions are used: *unconditional* and *conditional*. All observations are pooled in unconditional logistic regression. In conditional logistic regressions, the observations are stratified (e.g. based on matching pairs) and is thus efficient to control for bias.

Conditional logistic regressions were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) between exposures and the risk of developing Sjögren's syndrome in **Study I** and **Study II**.

3.5.2 Cox regression

The Cox proportional hazard model (or Cox regression) is designed for analysis of time to event data, commonly referred to as *survival analysis*. Models for survival analysis relate the time that passes before an event occurs to one or more variables that may be associated with that quantity of time.

Hazard rate is a generic term in survival analysis, used to describe the rate at which events are occurring. The *hazard function*, denoted as $\lambda_0(t)$, is the instantaneous event rate at time *t*, conditional on survival up to time *t*. Proportional hazard models, such as the Cox regression, estimate the risk of an event as a proportion of a baseline hazard, and can be expressed as:

$$\lambda(t\ |X) = \lambda_0(t) * \exp(\beta X)$$

Where $\lambda(t|X)$ denotes the hazard at time *t* for an individual with some value of the covariate X, which is a product of the baseline hazard, $\lambda_0(t)$, and a constant, $\exp(\beta X)$. Thus, a key concept is that proportional hazard models estimate ratios of hazard – *hazard ratios* (*HRs*) – that are constant over time, and that hazards are assumed to be proportional to each other over time.

Another central aspect of the Cox model, as opposed to other proportional hazard models, is that it makes no assumptions about the shape of underlying hazard function. Instead, the baseline hazard is allowed to vary freely, and the Cox model only estimates hazard ratios relative to it. As the hazard ratios are modelled parametrically, while the baseline hazard is not estimated, the Cox model is often referred to as "semi-parametric".

Cox regressions were used to estimate HRs and 95% CIs in **Study III–V.**

3.5.3 Multiple testing

In the context of statistical hypothesis testing, the p-value denotes the risk of conducting a *type I error*, i.e. falsely rejecting the null hypothesis. However, in the context of performing multiple tests, relying only on p-values becomes inappropriate. The more statistical tests that are performed, the greater is the possibility that one or more tests falsely rejects the null hypothesis. Thus, a multiple testing correction procedure may be needed to adjust statistical confidence measures based on the number of tests.

One of the more commonly known such corrections is the Bonferroni correction. The Bonferroni correction controls for the family wise error rate – that is, given a statistical significance threshold of α, the probability of one or more of the test falsely rejecting the null hypothesis is 1- α. There is however a trade-off when correcting of multiple testing, and minimizing the family-wise error rate may be deemed too strict. Another, less conservative, method is to conceptualize the rate of type I errors in multiple testing with the calculation of false discovery rates (FDR; often denoted as *q-values*). FDRs can be estimated with different methods, and may be interpreted as analogous to p-values where q-values denote the expected amount of false positives at a given threshold [183].

Q-values were calculated in **Study V** to account for multiple testing.

4 RESULTS

4.1 STUDY I: INFECTIONS PRECEDING SJÖGREN'S SYNDROME

Infections diagnosed in inpatient care or specialist outpatient care preceding Sjögren's syndrome diagnosis were recorded in 21% of the Sjögren's syndrome patients, and in 12% of the matched controls (OR 2.0, 95% CI 1.7–2.4) (**Study I, table 2**). Stratified on the anatomical site of infection, a history of infections of the respiratory tract, skin, and urogenital tract were significantly associated with an increased risk of developing Sjögren's syndrome.

The analyses were stratified by autoantibody status to include patients either positive for both Ro/SSA and La/SSB, or negative for both autoantibodies. Sjögren's syndrome patients with Ro/SSA and La/SSB had consistently higher exposure frequencies and odds ratios for any infection (OR 2.7, 95% CI 2.0–3.5), respiratory infections (OR 2.9, 95% CI 1.8–4.7), skin infections (OR 3.2, 95% CI 1.8–5.5), and urogenital infections (OR 2.7, 95% CI 1.7–4.2) compared to Sjögren's syndrome patients negative for Ro/SSA and La/SSB antibodies (**Study I, table 2**).

To investigate whether the risk of Sjögren's syndrome increases with the number of infections, infection events were categorized as 0, 1 or ≥ 2 and added to the model as a single categorical exposure variable. Interestingly, a dose-response relationship was observed for both respiratory and skin infections in Sjögren's syndrome patients positive for Ro/SSA and La/SSB antibodies, as an increasing number of infections associated with a higher odds ratio of Sjögren's syndrome (**Study I, figure 1**).

Sjögren's syndrome patients typically report disease symptoms multiple years before receiving their diagnosis. We therefore investigated whether applying a latency period of 3 or 7 years, thus taking into account only infections that had occurred more than 3 or 7 years prior to Sjögren's syndrome diagnosis, would modify the estimates. Interestingly, estimates were relatively stable as the latency period was extended (**Study I, figure 2**). In addition, a series of sensitivity tests were performed, in which the observed associations proved to be robust.

In summary, we observed an increased likelihood of developing Sjögren's syndrome following a history of infections. This association was most prominent for Ro/SSA and La/SSB autoantibody-positive patients.

4.2 STUDY II: SMOKING PRECEDING SJÖGREN'S SYNDROME

Thirty-seven percent of the Sjögren's syndrome patients reported having ever smoked prior to diagnosis date, compared to 44% in the control group and resulting in an odds ratio of 0.67 (95% CI: 0.55–0.81). The fraction of current-smokers at index date was 7% and 16% in cases and controls, respectively (OR 0.37, 95% CI: 0.26–0.53).

Next, in order to more thoroughly investigate smoking patterns preceding Sjögren's syndrome diagnosis, period prevalence of smoking during the five decades preceding index date were assessed separately. The fraction of ever smokers 49–40 years prior to Sjögren's

syndrome was not significantly different from controls (OR: 0.89, 95% CI 0.66–1.20). However, corresponding odds ratios for the following periods were consistently lower ($p<0.05$). Moreover, individuals who developed Sjögren's syndrome were more likely to discontinue smoking compared to controls (**Study II, figure 1**).

Analyses on smoking exposure were also stratified by the presence of Ro/SSA and/or La/SSB autoantibodies, where patterns of smoking were generally similar between autoantibody positive and negative patients.

Smoking exposure was also assessed in relation to HLA carriage. Alleles included for assessment were HLA-DRB1*03, which is associated with the production of Ro/SSA and La/SSB antibodies [60-62]; HLA-DRB1*15, associated with production of anti-Ro/SSA autoantibodies in Sjögren's syndrome and shown to increase the risk of MS in smokers [29, 62]; and HLA-DRB1*01/04/10, which interacts with smoking to increase the risk of RA [184]. Stratified by HLA haplotype presence in cases and controls, estimated odds ratios for ever smoking at the time of Sjögren's syndrome diagnosis was <1 regardless of HLA haplotype (**Study II, table 3**). Moreover, none of the investigated HLA alleles were found to have modulating effects on smoking in an interaction analysis (**Study II, figure 4**).

4.3 STUDY III: CARDIOVASCULAR DISEASE IN SJÖGREN'S SYNDROME

During a median follow-up of ten years, n=53 (5.6%) events of myocardial infarction, n=34 (3.6%) events of cerebral infarction, and n=50 (5.3%) events of venous thromboembolism were observed in the Sjögren's syndrome cohort. The corresponding incidence rates were 5.6, 3.6, and 5.4 events per 1,000 person-years, respectively. Compared to the general population controls, individuals with Sjögren's syndrome had a 60% higher risk of myocardial infarction, a 20% increased risk of cerebral infarction, and a two-fold risk of venous thromboembolism (**Study III**, **table 3**).

Analyses on the risk of events were also split into time-bands, defined as either 1–4, 5–9, and ≥10 years after Sjögren's syndrome diagnosis. For arterial cardiovascular disease - i.e. myocardial infarction and cerebral infarction - crude incidence rates and relative risks increased with age and disease duration (**Study III, figure 1 & figure 2**). Meanwhile, an elevated relative risk of venous thromboembolism was evident already at the time of Sjögren's syndrome diagnosis and in younger patients (**Study III, figure 3**).

Incidence and relative risk of cardiovascular disease was also estimated in strata based on Ro/SSA and La/SSB status. As for the global estimates of risk, the highest increases were observed strata of patients with Ro/SSA and/or La/SSB. This pattern was most pronounced for venous thromboembolism, where the HR was 3.1 (95% CI 1.9–4.8) for Ro/SSA and La/SSB positive patients, compared to 1.6 (95% CI 0.9–3.0) in patients without these antibodies. A similar pattern was also observed for cerebral infarction, although with fewer observed events; for myocardial infarction, estimates of relative risk across patient strata were of similar amplitude.

4.4 STUDY IV: MULTIPLE MYELOMA IN SJÖGREN'S SYNDROME

During a median follow-up of ten years, four events of multiple myeloma were identified in the Sjögren's syndrome cohort and n=14 events in the control cohort, corresponding to a HR of 2.9 (95% CI 0.9–8.7; **Study IV, table 1**). The median time to myeloma diagnosis in individuals experiencing the event was 3.8 and 10.2 years for Sjögren's syndrome patients and controls, respectively.

The analysis was also stratified based on Ro/SSA and/or La/SSB autoantibody status in Sjögren's syndrome patients, revealing that all events of multiple myeloma occurred in individuals with these autoantibodies. Three of the events were observed in individuals with both Ro/SSA and La/SSB, and corresponding to a HR of 6.2 (95% CI 1.5–26.0).

4.5 STUDY V: COMORBIDITY IN PATIENTS WITH ANTIBODY-MEDIATED CONGENITAL HEART BLOCK

The risk of disease in patients with CHB was initially analyzed in so-called "ICD blocks", each containing various related diagnoses, which were treated as dichotomous composite variables. The incidence of diagnoses relating to cardiovascular morbidity was significantly higher in patients with CHB compared to controls. Moreover, CHB was also associated with significantly increased HRs of diagnoses relating to infectious events, psychological disorders, and metabolic or inflammatory conditions (**Study V, table 2**). The incidence of disease in the same disease categories was assessed for siblings of the individuals with CHB compared to controls, where no significant associations were observed. Nonetheless, a trend indicating higher risks of diagnoses relating to cardiac diseases and inflammatory conditions was observed (**Study V, table 3**).

Next, a more granular investigation of comorbidity was performed by assessing the incidence of each 3-character ICD code in the above ICD blocks with significant HRs. Notably, CHB was associated with significantly increased HRs of multiple diagnoses of cardiac morbidity (**Study V**, **table 4**). Overall, n=20 (16.8%) patients with CHB were diagnosed with cardiomyopathy and/or heart failure during the observation period (HR 70.0, 95% CI 20.8–235.4). Nine (7.6%) patients with CHB developed atrial fibrillation and flutter (HR 46.7, 95% CI 10.1–216.1). N=4 (3.4%) patients with CHB were diagnosed with a cerebral infarction, compared to one individual (0.08%) in the control group (HR 39.9, 95% CI 4.5–357.3).

A dichotomous composite variable of autoimmune diagnoses was created to assess the aggregated risk of such disorders in individuals with CHB and their siblings (see **Study V, supplementary table S5** for definition). The age-wise accumulation of diagnoses was calculated using a Nelson-Aalen estimator, and Cox models were fitted to compute statistical inference of differences in incidence between the groups (**Study V, figure 2**). Both individuals with CHB and their siblings were significantly more likely to be diagnosed with autoimmune disorders compared to their respective controls, with HRs of 5.7 (95% CI 2.8–11.6) and 3.6 (1.7–8.0), respectively. The risk of autoimmune diseases in individuals with CHB was not significantly different from their siblings (HR 1.7, 95% CI 0.7–4.1).

5 DISCUSSION

5.1 ENVIRONMENTAL RISK FACTORS IN SJÖGREN'S SYNDROME

The precise nature of how and why autoimmune diseases develop is not known, yet it is evident that the process is dependent on both genetic and environmental factors. Both infectious events and smoking have repeatedly been proposed as potential causative agents in the development of autoimmunity. In **Study I** and **Study II**, we conducted two case-control studies to investigate how these two factors associate with Sjögren's syndrome.

With the known time lag between patient-reported symptom onset and clinical diagnosis, the temporal relationship between exposure and the diagnosis of Sjögren's syndrome was of special interest. Although epidemiological investigations ultimately cannot prove causality, findings of exposures predating presumed symptom debut may to a higher degree support a causal relationship.

Furthermore, differences in exposure profiles between Ro/SSA and La/SSB positive and negative Sjögren's syndrome patients were of interest. The presence of Ro/SSA and La/SSB autoantibodies demark a patient group with distinct MHC genotypes, whereas genetic features in Sjögren's syndrome patients without these autoantibodies have not been demonstrated to significantly differ from the population at large [62, 185-187]. Given the differences in genetic constitution, we were intrigued to examine whether exposure data prior to diagnosis would differ between these two groups.

5.1.1 Study I

A prominent and robust association between a history of infections and the development of Sjögren's syndrome was observed. Overall, a history of infection diagnosed in inpatient or non-primary outpatient was associated with a two-fold increased risk of Sjögren's syndrome, in line with the findings of a previous study based on register data in Denmark [188]. Stratified on anatomical location, infections in the skin, respiratory and urogenital tract were associated with significantly increased risk of Sjögren's syndrome.

Stratified on serological profile, a central finding from the study was the consistently more prominent association between infection and Ro/SSA and La/SSB positive Sjögren's syndrome. Additionally, a dose-response relationship between infections and risk of developing Sjögren's syndrome with Ro/SSA and La/SSB antibodies was observed. Indeed, repeated infectious events evoking the adaptive arm of the immune system may facilitate the breaking of immunological tolerance.

The more prominent association in antibody-positive Sjögren's syndrome patients is especially interesting given previous studies demonstrating a link between Ro/SSA orthologs from bacteria from skin and mucosal tissue, and the activation of the antigen-specific immune system in experimental autoimmune *in vitro* and *in vivo* models [67]. However, in contrast to other research on infections triggering autoimmunity, our findings do not suggest the presence of any specific infectious agent that through molecular mimicry triggers the development of autoimmunity. Rather, it may be the initiation of inflammation, such as type I IFN, and exposure of autoantigens that contributes to an erroneous response

in lymphocytes in individuals with a genetically higher propensity to develop autoimmunity.

As opposed to infections contributing to the development of Sjögren's syndrome, individuals progressing to a clinically overt disease may become more susceptible to infections from local aberrations in immunity and dysfunctional mucociliary clearance [189, 190]. Additionally, disease-specific symptoms and the presence of Ro/SSA and La/SSB antibodies are known to precede the diagnosis by multiple years [191, 192]. We attempted to mitigate the effects from such reversed causality by excluding infections occurring within up to seven years prior to Sjögren's syndrome diagnosis. Estimates for individuals who progressed to Ro/SSA and La/SSB positive Sjögren's syndrome were however largely unaltered, supporting the hypothesis of infectious events having a causal effect on the development of Sjögren's syndrome.

Another potential explanation for the findings, for which we were unable to control for, is that individuals who develop Sjögren's syndrome may be genetically more prone to develop more-than-moderate inflammation upon infection and thus have a higher propensity to be diagnosed with infections in an inpatient or outpatient setting.

In summary, a history of infections is associated with a prominent and robust association with the development of Sjögren's syndrome, and is especially prominent in patients developing Ro/SSA and La/SSB antibodies. In conjunction with the known pathophysiological mechanisms of the disease, as well as findings from previous research, infections appear as a plausible contributing cause to the development of Sjögren's syndrome.

5.1.2 Study II

Smoking did not emerge as a risk factor for the development of Sjögren's syndrome, as a lower frequency of smoking preceding Sjögren's syndrome diagnosis was observed compared to controls. However, longitudinal assessment of smoking patterns revealed that individuals who developed Sjögren's syndrome smoked to an extent similar to as the population in early life, but where significantly more prone to stop smoking. This finding is consistent with previous studies reporting a decreased prevalence of smoking in Sjögren's syndrome, as well as a smaller prospective study in which discontinuation of smoking was linked to an increased risk of developing the disease [23, 146, 193, 194]. Notably, this shift in smoking habits was observed earlier than the average timing of self-reported symptom onset of Sjögren's syndrome [195]. While the data allow for different interpretations, it may reflect very early disease symptoms and underlying pathological changes.

Patterns of cigarette smoking were also relatively similar regardless of Ro/SSA and/or La/SSB antibody status at time of Sjögren's syndrome diagnosis. Given the differences in genetic risk variants, age distribution, and clinical disease course between autoantibody positive and negative patients [62, 84, 187, 196], different findings may have been anticipated. Nonetheless, the common finding of both groups having a higher predisposition to discontinue smoking speaks for the hypothesis of early symptoms of disease driving this trend.

The finding of smoking not increasing the risk of developing Sjögren's syndrome is in contrast to the knowledge on role of cigarette smoking in other autoimmune diseases such as SLE, RA, MS, and Crohn's disease [197, 198]. However, smoking has been associated with a reduced risk of developing ulcerative colitis as well as Behçet's disease [199-201]. For ulcerative colitis, cessation of smoking has both been associated with development of the disease as well as worsening of symptoms [199]. The seemingly opposite effects from smoking on the inflammatory bowel diseases Crohn's disease and ulcerative colitis could potentially be explained by distinct cytokine profiles in the two disease entities, driven by Th1 cells and Th2 cells, respectively [202]. In a murine model, smoking exposure was associated with an increase in IL-33, associated with both ulcerative colitis and Sjögren's syndrome [203, 204], but however altering the immune response from IL-33 from Th2 to Th1 [205], thus providing a speculative mechanistic explanation as to how smoking could decrease the risk of inflammatory disease. Importantly, it should however be noted that cigarette smoking has not been shown to alter the natural course of ulcerative colitis [199].

Given the previous data of interaction effects between smoking exposure and MHC alleles, we investigated the relationship between these variables and the development of Sjögren's syndrome. Perhaps not surprisingly given an overall lower exposure to smoking in Sjögren's syndrome patients, an interaction effect between smoking and risk-associated HLA was not observed. This finding is in contrast to previous studies in RA and MS, where gene-environment interactions between HLA and smoking have been identified [4, 5].

In conclusion, smoking exposure preceding Sjögren's syndrome diagnosis was lower compared to controls. Observed patterns of smoking habits indicate that individuals who develop Sjögren's syndrome smoke equally as the general population in early life, but then are more prone to stop. We interpret this finding to potentially reflect early pathological changes, highlighting the progressive development of the disease.

5.2 COMORBIDITY IN PATIENTS WITH SJÖGREN'S SYNDROME

Chronic inflammation is increasingly recognized as central element in contributing to a wide range of pathological processes in the body. Still, what types of inflammation or patient groups may result in clinically overt disorders is not well characterized.

The prevention of disease is unambiguously preferable to treating already clinically manifest illness. In order to effectively direct preventive efforts to groups of individuals, reliable estimates of disease occurrence – and what subpopulations they apply to – are pivotal. The spectrum of comorbidity presenting in patients with primary Sjögren's syndrome has not been thoroughly studied in large cohorts of clinically verified patients. Moreover, most investigations studying morbidity associated with Sjögren's syndrome have seldom discriminated patients by relevant biomarkers such as Ro/SSA and La/SSB, whose presence mark two genetically and clinically distinct subgroups.

5.2.1 Study III

Inflammation is mechanistically linked to cardiovascular disease development, and increased morbidity and premature mortality related to cardiovascular events have

previously been reported for RA and SLE [135-137]. In Sjögren's syndrome, relatively few studies have addressed the risk of cardiovascular disease, and with inconsistent results [141, 144, 145, 148-152]. Our findings suggest that individuals with primary Sjögren's syndrome have substantially increased risks of cardiovascular disease in the form of myocardial infarction, cerebral infarction, and venous thromboembolism.

The most prominent finding was observed for venous thromboembolism, for which patients with Sjögren's syndrome had a two-fold increased risk compared to the general population, and was in accordance with results from a recent meta-analysis [143]. However, previously unreported, our findings indicate that Sjögren's syndrome patients with both Ro/SSA and La/SSB antibodies have a 3-fold risk. The increased risk of venous thromboembolism in Sjögren's syndrome patients has been suggested to arise from chronic inflammation, which increases coagulability [206], and is also observed in other systemic autoimmune diseases [141, 143, 207, 208]. Indeed, the more prominent systemic inflammation associated with Sjögren's syndrome with Ro/SSA and La/SSB antibodies may explain the higher risk of venous thromboembolism in this group of patients.

Patients with Sjögren's syndrome also appear to have an increased relative risk of arterial cardiovascular disease. As opposed to our findings for venous thromboembolism, this risk appears to be initially low, but increases with disease duration and age. This observation is consistent with the progressive buildup of atherosclerotic plaques, which is widely considered an inflammatory process and the main underlying cause of arterial ischemic diseases. Notably, higher frequencies of subclinical atherosclerosis have been described in patients with Sjögren's syndrome [147, 209-211], as has been suggested to correlate with disease duration [212]. Also for arterial diseases, a trend indicating higher risk of disease in autoantibody-positive Sjögren's syndrome was observed. Interestingly, Ro/SSA and La/SSB antibodies have been linked with subclinical atherosclerosis and endothelial dysfunction in Sjögren's syndrome patients [209-211]. Our findings are thus consistent with previous reports, and suggest that their findings also translate into clinically overt conditions.

Our findings on the risk of arterial cardiovascular disease is lower compared to what has been reported in meta analyses of RA and SLE, where pooled relative risks for have been estimated to 1.7 and 3.0 for myocardial infarction, and 1.4 and 2.0 for cerebrovascular accidents, respectively [207, 213]. Moreover, the absence of increased risk of arterial cardiovascular in early disease is also in contrast to findings in RA and SLE, where increased risk is observed also in younger patients [214, 215]. Arguably, this discrepancy may be explained by the more severe inflammation in such rheumatic disorders, as well as an earlier onset of disease.

Noteworthy variables whose effects on cardiovascular disease we were unable to assess were lifestyle factors, such as sedentary behavior, and other comorbidities. Previous studies have suggested that individuals with Sjögren's syndrome have an increased frequency of traditional risk factors such as serum lipid aberrations and hypertension [144-146]. Additionally, the impact of antiphospholipid antibodies would be interesting to investigate.

5.2.2 Study IV

An increased risk of lymphoproliferative diseases in Sjögren's syndrome patients, mainly in the form of non-Hodgkin's lymphomas, is well established. However, while autoimmune diseases in general have been linked to an increased risk of multiple myeloma [216, 217], studies examining this association in primary Sjögren's syndrome are few and have been inconsistent. Case-control studies of multiple myeloma have not identified Sjögren's syndrome as a risk factor for multiple myeloma [218, 219]; meanwhile, cohort studies of Sjögren's syndrome patients have resulted in highly variable estimates of standard incidence ratios, ranging between a non-significant 3.4 and a significant 37.9 [106-108]. A recent meta-analysis calculated a pooled relative risk of 2.37 (95% CI 0.70–8.02) [216]. While with no conclusive findings, our study implicates that there might be an increased risk of multiple myeloma in individuals with Sjögren's syndrome. However, this risk appears to be confined to individuals with Ro/SSA and La/SSB antibodies.

Analogously, while the risk of lymphoma development in Sjögren's syndrome has been described to increase with disease duration [89], our results indicate a potentially short time span between Sjögren's syndrome diagnosis and multiple myeloma. Given the hypothesis of chronic inflammation as the main driver of malignant transformation, this finding was not expected. However, it could in part be explained by the generally long time period between the initiation of Sjögren's-specific pathological changes and diagnosis when the syndrome becomes clinically overt. Naturally, the observed temporal proximity between Sjögren's syndrome diagnosis and myeloma diagnosis was affected from a limited follow-up time.

Notably, all observed events of multiple myeloma in Sjögren's syndrome patients occurred in individuals with Ro/SSA and/or La/SSB autoantibodies. With the very limited number of events, this outcome and estimated risk should be interpreted with caution. However, given the more prominent inflammation and B-cell activation in autoantibody positive Sjögren's syndrome, the clustering of events in this subpopulation is not unanticipated. In addition, the observation of higher morbidity in individuals with Ro/SSA and La/SSB antibodies is concordant with our findings in **Study III**.

In conclusion, our findings suggest that there is an increased risk of multiple myeloma in Sjögren's syndrome patients. Ro/SSA and La/SSB autoantibodies appear to demark the population with elevated risk, although further studies are warranted to examine their capacity as predictive markers.

5.3 COMORBIDITY IN PATIENTS WITH CONGENITAL HEART BLOCK

Studies examining morbidity in individuals with CHB during later life are lacking, much due to the rareness of the condition. In an attempt to holistically assess the panorama of morbidity, we performed an epidemiological cohort study where morbidity was assessed in an exploratory fashion.

Individuals with CHB appear to have an increased risk of cardiovascular comorbidities in later life, such as heart failure and arrhythmias, consistent with previous findings [165, 220-223]. Moreover, our findings also indicate an increased risk of morbidity in the form of cerebral infarction.

The condition was also associated with a higher incidence rate of infectious diseases. The reason behind this observation is not elucidated, and may be multifactorial. While infections secondary to pacemaker surgery appeared to explain some of the risk, reporting bias may also contribute to our observations. Additionally, the increased prevalence of prematurity reported in individuals with CHB [153, 224], is also associated with an increased risk of infections [225]. Our findings also suggest that CHB is associated with an increased frequency of psychological developmental disorders, which is also is consistent with reports from previous studies [167, 168].

Lastly, patients with CHB, as well as their siblings, had a higher risk of developing inflammatory diseases. Considering the presence of maternal Ro/SSA antibodies present in all individuals, implying a genetic predisposition for autoimmunity, this observation was not unexpected given familial aggregation of such diseases.

In summary, our study suggests a considerable burden of comorbidity for patients with CHB, most present within cardiovascular, infectious and inflammatory disorders, the latter also shared by their siblings.

5.4 LIMITATIONS

The studies have limitations to consider, of which some general aspects are discussed below.

5.4.1 Misclassification of exposure

As the immunoassays used to identify Ro/SSA and La/SSB antibodies may have changed over calendar time and hospital, some misclassification with regard to serological profile might be anticipated. This error rate is however presumably low and non-differentiated, diluting observed differences between antibody positive and negative Sjögren's syndrome patients.

Individuals progressing in the development of Sjögren's syndrome may inherently have more exposure to health-care as their disease is manifested, thus being more likely to be diagnosed with conditions otherwise not recorded by physicians. This phenomenon could therefore incur exposure misclassification bias in **Study I**, inflating the association between infectious events and the development of the disease. In an attempt to mitigate such bias, we tried applying longer latency periods, as well as adjusting for previous health-care consumption density.

The questionnaire-based **Study II** was inherently vulnerable to recall bias, where patients with Sjögren's syndrome may have over-reported exposure to cigarette smoking. The extent of any such misclassification bias was not estimated, but is assumed not to be of major magnitude, partly due to patients reporting having smoked to a smaller extent than comparators, as well as the questionnaire not stipulating any hypothesis and including questions un multiple lifestyle habits. Additionally, the discrepancy in definition of index date between cases and controls, where substantial time between index date and date of

responding to the questionnaire had elapsed for cases, may constitute an additional source of bias, whose eventual size and direction we were unable to informedly speculate about.

5.4.2 Misclassification of outcome

The register-based outcome variables of cardiovascular morbidity investigated in **Study III** have been previously shown to have high positive predictive values, and given the population based coverage of the NPR, may be assumed to hold a high sensitivity as well. Thus, the magnitude of eventual surveillance bias is probably minimal. Likewise, the Swedish Cancer Register, used to identify morbidity in **Study IV**, is also a high-quality register with presumably good validity and exhaustiveness, minimizing the risk of bias derived from misclassification.

In contrast, the assessment of morbidity in **Study V** was performed in an exploratory fashion, where some outcome variables most likely will have been subject to surveillance bias and thus inflated observed associations.

5.4.3 Missing variables

Residual and unmeasured confounding can cause associations that are not causal, and there were naturally multiple factors for whose effect we were unable to fully control for or investigate.

Data from primary care would have provided a more exhaustive coverage of morbidity, and may have provided important information in some analyses. Relatedly, we were unable to control for the effects for prescribed drugs, although inclusion of such variables would most likely be subject to confounding by indication. Lastly, we did not control for the effects from life-style factors.

5.4.4 Observation periods

The average amount of observational time available per subject may, by current standards, be considered a strength of the studies included in this thesis. However, as the mean observation or follow-up time was typically one decennium, it may have hampered global estimations of associations. Relatedly, given the apparent differences in age between autoantibody positive and negative Sjögren's syndrome patients, observational time was not similarly distributed across the subgroups on the age timescale.

Distorting effects from the left-truncation incurred by inclusion of prevalent cases at start of follow-up in **Study V**, should not be neglected. Nonetheless, the inclusion of prevalent cases was motivated from a pragmatic perspective, as they provided follow-up time of individuals with CHB during older ages.

5.5 STRENGTHS

The studies also have several unique features and strengths. A key strength of **Study I–IV** relates to the investigated individuals with Sjögren's syndrome having a validated diagnosis according to the internationally accepted AECG criteria. This feature assured that the intended group of patients was studied, contributing to the generalizability of our findings. Additionally, we were able to stratify the patients based on the Ro/SSA and La/SSB antibody status, whose presence or absence discriminate between distinct patient subgroups. Notably, while the global estimates for Sjögren's syndrome patients in **Study II**, **III** and **IV** were in proximity with those of previous investigations and meta-analyses, the stratification by autoantibody profile revealed strikingly different properties in patient subgroups. Indeed, the stratification of patients based on serological profile constitute one of the key contributions of the work conducted within this thesis.

CHB is a most rare condition, lacking systemic examination in literature. Therefore, the cohort of patients in **Study V**, although small, presented an unequaled opportunity to assess comorbidity associating with this disorder.

The usage of matched and randomly selected controls from the general population unambiguously contributed to creating valid comparator groups, benefiting the internal validity of the findings. Lastly, the population-based registers used to identify morbidity have excellent coverage and are prospectively collected, thus minimizing the risk of recall and detection bias, as well as non-response bias.

6 CONCLUSIONS

From the works presented in this thesis, the main findings can be summarized in the following points:

- Infections precede the development of primary Sjögren's syndrome. This association is robust, and more prominent in individuals with Ro/SSA and La/SSB autoantibodies. These findings may be interpreted as infections indeed contributing to the development of the disease.

- Smoking could not be linked to an increased risk of developing Sjögren's syndrome. As the prevalence of smoking decreased multiple years prior to the diagnosis of the disease, regardless of immunopathological profile, it may be interpreted as early symptoms of the syndrome affecting smoking habits.

- The burden of cardiovascular disease in patients with primary Sjögren's syndrome is significant. The presence of Ro/SSA and La/SSB autoantibodies appear to demark the population with the highest risk, especially with regard to venous thromboembolism.

- Primary Sjögren's syndrome appears to be associated with an increased risk of multiple myeloma, which is also more prevalent in individuals with Ro/SSA and La/SSB autoantibodies.

- The burden of morbidity associated with congential heart block is substantial. Individuals with the condition appear to have an increased risk of cardiovascular, infectious and inflammatory disorders.

7 FUTURE PERSPECTIVES

While the studies presented in this thesis provide new insights, they also open up for new research prospects. Some of them are discussed briefly below.

Identification of novel biomarkers for patient stratification

Patient stratification by the presence of specific autoantibodies – Ro/SSA and La/SSB – revealed pronounced differences in subpopulations of Sjögren's syndrome individuals. However, as the autoantibodies are merely proxy variables of the underlying mechanistic and inflammatory environment in these patients, a more granular assessment of the proteomic state and whether such biomarkers more precisely can predict long-term complications and comorbidity would be interesting to investigate. Indeed, a more precise clustering of patients could help identify individuals who will benefit from a more aggressive treatment strategy and frequent monitoring.

More specifically related to studies presented in this thesis, predictors of cardiovascular and hematological morbidity emerge as plausible fields for further assessment.

Based on the findings from **Study III**, it is evident that patients with Sjögren's syndrome have a substantially increased risk of cardiovascular morbidity. From the observation of risk being higher in Ro/SSA and/or La/SSB positive patients, it appears likely that inflammation is central in driving this hazard. Assessment of vascular tissue and coagulation markers in Sjögren's syndrome patients may identify what elements of cardiovascular homeostasis are disrupted and associated with clinically overt disease. The identification of such markers may therefore serve as potential prognostic markers as well as targets for therapeutic interventions.

Contradictory to the current standpoint in the literature on lymphoma predictors in Sjögren's syndrome [96], preliminary investigation on the cohort in **Study IV** indicates that Ro/SSA and La/SSB autoantibodies indeed are associated with the development of lymphomas in general. Assessment of this relationship is therefore relevant. Relatedly, an exploratory identification of lymphoma predictors using high-throughput assays may also provide valuable findings.

Gene-environment interactions in infectious diseases

The association between infectious events and the succeeding development of Sjögren's syndrome in **Study I** was especially prominent in individuals with Ro/SSA and La/SSB autoantibodies, implying a potential genetic component. Studies examining the presence of an eventual gene-environment interaction between infections and Sjögren's-associated genes could provide further insights into the etiopathogenesis of the disease. Moreover, such insights could aid in identifying individuals who are prone to develop the disease, and how development of clinically overt disease may ultimately be prevented.

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