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Sjögren's syndrome: epidemiological risk factors and biomarkers

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Sjögren's syndrome: epidemiological risk factors and biomarkers

Sjögren’s syndrome: epidemiological risk factors and biomarkers

Peter Olsson

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DOCTORAL DISSERTATION
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Faculty opponent
Associate professor Marja Pertovaara
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<tr><td colspan="4">Abstract
Primary Sjögren’s syndrome (pSS) is an autoimmune disease, primarliy affecting women, characterised by inflammation and destruction of exocrine glands with a prevalence of 0.01-0.09%. About one-third of the patients also suffer from extraglandular manifestations e.g. inflammation in the lungs, kidneys, skin or nerves. Apart from dyness symptoms, patients often suffer from fatigue and pain. Little is known about epidemiological risk factors other than a negative association between smoking and pSS.
The aims of this work was to identify epidemiological risk factors for the development of pSS and to analyse biomarkers (cytokines) in relation to found epidemiological risk factors. In Study I, the Malmö Sjögren’s syndrome registry (MSSR) was linked to two large population based prospective health surveys: the Malmö Preventive Medicine Programme (MPMP) and the Malmö Diet and Cancer Study (MDCS). The prediagnostic data aquired from the health studies revealed a negative association between current smoking and a subsequent development of pSS. On the other hand, being a former smoker at the time of the health surveys was associated with a higher risk of later developing pSS.
pSS patients are frequently rheumatoid factor (RF) positive. RF can cross-bind the antibodies used in sandwich Enzyme-Linked Immunosorbent Assays (ELISAs), which is common method for analysing cytokine levels, and cause erroneous results. Study II included an analysis investigating the potential interaction by RF, and/or heterophilic antibodies, in a bead-ELISA platform using sera from four RF positive patients with rheumatoid arthritis and two RF positive patients with pSS. Erroneous high cytokine values were found in all six patients in almost all cytokine measured and the erroneous values were reduced when using a commercial blocker of heterophilic antibodies/RF.
Two studies were performed to further investigate the relation between smoking and pSS. In Study III, smoking status was investigated in relation to disease activity and cytokine expression [Interleukin (IL)-1β, IL-2, IL-3, IL-6, IL-8, IL-10, IL-12 (p70), IL-17, IL-18, IL-33, Interferon (IFN)α2a, IFNγ, Epidermal growth factor (EGF), Regulated upon Activation, Normal T-cell Expressed and presumably Secreted (RANTES), FAS-ligand, B-cell activating factor, Tumor Necrosis (TNF)-α, and Transforming growth factor-β1 (TGF-β1)] in 51 consecutive pSS patients. In Study IV, smoking status and cigarette consumption was investigated in relation disease activity and presence of an type I interferon (IFN) signature in 90 consecutive patients. A type I IFN signature was defined as an increased expression of five type I IFN related genes (IFI44, IFI44L, IFIT1, IFIT3 and MxA), analysed using reverse transcriptase polymerase chain reaction (RT-PCR). No associations between smoking status and cytokine expression, presence of an IFN signature or disease activity [as measured by EULAR Sjögren’s Syndorme Disease Activity Index (ESSDAI)] were found. However, high cigarette consumtion (pack-years ≥ 8.8 years) was associated to a higher burden of symptoms [as measured by EULAR Sjögren’s Syndrome Patient Reported Index (ESSPRI)]
In conclusion we found a negative associaton between current cigarette smoking and subsequent pSS diagnosis but we did not find any associations between cigarette smoking and disease activity, cytokine expression or presens of a type I IFN signature in pSS patients. In both Study III and Study IV current smokers were few, making it difficult to draw any firm conclusions regarding this group.</td></tr>
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Sjögren’s syndrome: epidemiological risk factors and biomarkers

Peter Olsson

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To Sara, Astrid, Elin and Allan

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List of publications

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals. Papers I–III have been reprinted by permission of the publishers.

I) Cigarette smoking and the risk of primary Sjögren's syndrome: a nested case-control study
Olsson P, Turesson C, Mandl T, Jacobsson L, Theander E.
Arthritis Research & Therapy 2017;19:50

II) Multiplex cytokine analyses in patients with rheumatoid arthritis require use of agents blocking heterophilic antibody activity.
Olsson P, Theander E, Bergström U, Jovinge S, Jacobsson L, Turesson C.
Scandinavian Journal of Rheumatology 2017;46:1–10

III) Smoking, disease characteristics and serum cytokine levels in patients with primary Sjögren's syndrome.
Olsson P, Skogstrand K, Nilsson A, Turesson C, Jacobsson L, Theander E, Houen G, Mandl T.
Rheumatology International 2018;38:1503–10

IV) Associations between cigarette smoking and disease burden, symptoms and type I interferon expression in primary Sjögren's syndrome.
Olsson P, Bodewes I, Nilsson A, Turesson C, Jacobsson L, Theander E, Versnel M, Mandl T.
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I have also contributed to the following papers not included in the thesis:

I) Prediction of Sjogren's Syndrome Years Before Diagnosis and Identification of Patients With Early Onset and Severe Disease Course by Autoantibody Profiling
Theander E, Jonsson R, Sjostrom B, Brokstad K, Olsson P, Henriksson G
Arthritis & Rheumatology 2015;67(9):2427–36

II) Work Disability in Newly Diagnosed Patients with Primary Sjogren Syndrome
Mandl T, Jorgensen TS, Skougaard M, Olsson P, Kristensen LE
The Journal of Rheumatology 2017;44(2):209–15

III) Severe intestinal dysbiosis is prevalent in primary Sjogren's syndrome and is associated with systemic disease activity
Mandl T, Marsal J, Olsson P, Ohlsson B, Andreasson K
Arthritis Research & Therapy 2017;19(1):237

IV) Respiratory symptoms are poor predictors of concomitant chronic obstructive pulmonary disease in patients with primary Sjogren's syndrome
Strevens Bolmgren V, Olsson P, Wollmer P, Hesselstrand R, Mandl T
Rheumatology International 2017;37(5):813–8

V) Autoantibodies to Cytosolic 5'-Nucleotidase 1A in Primary Sjogren's Syndrome and Systemic Lupus Erythematosus
Rietveld A, van den Hoogen LL, Bizzaro N, Blokland SLM, Dahnrich C, Gottenberg JE, Houen G, Johannsen N, Mandl T, Meyer A, Nielsen CT, Olsson P, van Roon J, Schlumberger W, van Engelen BGM, Saris CGJ., Pruijn GJM
Frontiers in Immunol. 2018;9:1200

VI) Increased B-cell activating factor, interleukin-6, and interleukin-8 in induced sputum from primary Sjogren's syndrome patients
Nilsson AM, Tufvesson E, Hesselstrand R, Olsson P, Wollmer P, Mandl T.
Scandinavian Journal of Rheumatology 2019;48(2):149–56.

VII) How immunological profile drives clinical phenotype of primary Sjogren's syndrome at diagnosis: analysis of 10,500 patients (Sjogren Big Data Project)
Brito-Zeron P, Acar-Denizli N, Ng WF, Zeher M, Rasmussen A, Mandl T, Seror R, Li X, Baldini C, Gottenberg JE, Danda D, Quartuccio L, Priori R, Hernandez-Molina G, Armagan B, Kruize, AA, Kwok, SK, Kvarnstrom M, Praprotnik S, Sene D, Bartoloni E, Solans R, Rischmueller M, Suzuki Y, Isenberg D, Valim V, Wiland P, Nordmark G, Fraile G, Bootsma H, Nakamura T, Giacomelli R, Devauchelle-

Pensec V, Knopf A, Bombardieri M, Trevisani VF, Hammenfors D, Pasoto SG, Retamozo S, Gheita TA, Atzeni F, Morel J, Vollenveider C, Horvath IF, Sivils KL, Olsson P, De Vita S, Sanchez-Guerrero J, Kilic L, Wahren-Herlenius M, Mariette X, Ramos-Casals M
Clinical and Experimental Rheumatology 2018;36 Suppl 112(3):102–12

Abbreviations

AECG:	American-European Consensus Group
ANA:	Antinuclear antibodies
ACR:	American College of Rheumatology
BAFF:	B-cell activating factor
BLK:	B-lymphocyte kinase
BTK:	Bruton's tyrosine kinase
C3:	Complement component 3
C4:	Complement component 4
CMV:	Cytomegalovirus
CNS:	Central nervous system
COPD:	Chronic obstructive pulmonary disease
CRP:	C-reactive protein
CT:	Computerised tomography
CXCR5:	Chemokine (C-X-C-motif) receptor 5
DAS28:	Disease activity score based on 28 joints
DC:	Dendritic cell
DNA:	deoxyribonucleic acid
DSR:	DNA sensing receptor
EBF-1:	Early B-cell factor-1
EBV:	Epstein Barr virus
EGF:	Epidermal growth factor
EGM:	Extraglandular manifestations
ELISA:	Enzyme-linked immunosorbent assay
ESSDAI:	EULAR Sjögren's syndrome disease activity index
ESSPRI:	EULAR Sjögren's syndrome patient reported index
ESR:	Erythrocyte sedimentation rate
EULAR:	European League Against Rheumatism
Fab:	Fragment antigen-binding
Fc:	Fragment crystallisable
FcγRII:	Fc-γ receptor II
FI:	Fluorescence intensity
GOLD:	Global initiative for lung disease
HAQ-DI:	Health assessment questionnaire disability index
HHV:	Human herpes virus
HIV:	Human immunodeficiency virus
HLA:	Human leukocyte antigen
iDC:	Immature dendritic cell
IFI44:	Interferon induced protein 44
IFI44L:	Interferon induced protein 44 like

IFIT:	Interferon induced protein with tetratricopeptide repeats
IFN:	Interferon
IFNAR:	Interferon-α/β receptor
IFNγ:	Interferon gamma
Ig:	Immunoglobulin
IL:	Interleukin
ILD:	Interstitial lung disease
IRF5:	Interferon regulatory factor 5
JAK:	Janus Kinase
kDa:	kilodalton
MCTD:	Mixed connective tissue disease
NF-κB:	Nuclear factor κB
NK-cell:	Natural killer cell
OAS1:	2´-5´-oligoadenylate synthase 1
pDC:	Plasmacytoid dendritic cell
PNS:	Peripheral nervous system
PRR:	Pattern recognition receptor
pSS:	Primary Sjögren's syndrome
RANTES:	Regulated upon Activation, Normal T-cell expressed and presumably Secreted
RF:	Rheumatoid factor
RLR:	RIG-1 like receptor
RIG-1:	Retinoic acid inducible gene 1
SLE:	Systemic lupus erythematosus
STAT4:	Signal transducer and activator of transcription 4
SSc:	Systemic sclerosis
SYK:	Spleen tyrosine kinase
TLR:	Toll-like receptor
TNIP1:	Tumour necrosis factor α induced protein 3 interacting protein 1
TNF:	Tumour necrosis factor
TNFAIP3:	Tumour necrosis factor alpha induced protein 3
TNFSF4:	Tumour necrosis factor superfamily 4
VAS:	Visual analogue scale

Introduction

Background

Historical notes

Sjögren's syndrome is named after the Swedish ophthalmologist Henrik Sjögren (1899–1986) who described 19 women with severe dryness and arthralgia in his dissertation "Zur Kenntnis der Keratoconjunctivitis sicca" in 1933 (1). Some have argued that it should be called Morbus Gougerot instead, after the French physician Henri Gougerot (1881–1955) who in 1926 described three patients with symptoms typical of the disease in: "*Insuffisance progressive et atrophie des glandes salivaires et muqueuses de la bouche, des conjonctives (et parfois des muqueuses nasale, laryngé et vulvaire) sécheresse de la bouche, des conjonctives, etc*" (2). Others have pointed out the contributions made by Mikulicz, Hadden and Mulock Houwer (3-5). Although it is possible that they all described patients with what is now called Sjögren's syndrome, Henrik Sjögren was the first to establish the systemic character of the disease, which was an important observation.

Clinical description of pSS

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease, causing lymphocytic infiltration and destruction of the exocrine glands, predominantly the salivary and lacrimal glands, but also the exocrine glands in the nasal cavity, pharynx, airways and, in women, the vagina. The inflammation and destruction of the glands results in mucosal dryness (6). Pronounced caries is common amongst patients, as is candida infection, due the dry mucous membranes. Affection of other organs, often termed extraglandular manifestations (EGM), is seen in approximately one third of patients, entailing pulmonary, kidney, skin and nervous system involvement (7) although the affection is usually not as severe as in other systemic rheumatological diseases. Many patients suffer from a severe fatigue (8) and a substantial fraction of patients also suffer from fibromyalgia (9) resulting in an increased rate of work disability amongst pSS patients (10). pSS is a multifaceted disease and the possible complications are summarised in Table 1. In addition, patients with pSS have an up to 16-fold increased risk of non-Hodgkin lymphoma, mostly MALT-lymphoma, due to the chronic activation of lymphocytes (11).

Box 1 | **Organ-by-organ systemic manifestations**

Oral symptoms
- Hyposalivation
- Soreness
- Adherence of food to the mucosa
- Dysphagia
- Difficulties in speaking or eating
- Dental caries
- Oral candidiasis

Ocular symptoms
- Insufficient tears
- Inability to tear
- Foreign-body sensation
- Conjunctival inflammation (keratoconjunctivitis sicca)
- Eye fatigue
- Decreased visual acuity
- Blepharitis
- Bacterial keratitis

General symptoms
- Fatigue
- Chronic pain
- Low-grade fever
- Weight loss

Lymph node complications
- Reactive multiple lymphadenopathy (swelling of the lymph nodes)
- Lymphoproliferative complications

Cutaneous complications
- Cutaneous vasculitis (10% of patients)
- Purpura
- Cutaneous ulcers*
- Annular erythema (9% of patients)
- Xerosis cutis (abnormally dry skin; 23–68% of patients)

Articular and muscle complications
- Arthralgias (joint pain; 60–70% of patients)
- Non-erosive symmetric arthritis
- Subclinical synovitis (20–30% of patients)
- Jaccoud arthropathy (non-erosive joint disorder)‡
- Myalgias (20–40% of patients)
- Myositis‡

Pulmonary complications
- Chronic obstructive lung disease
- Bronchiectasis
- Interstitial lung diseases*
- Pleuritis‡

Cardiovascular complications
- Raynaud phenomenon (13% of patients)
- Pericarditis‡
- Pulmonary arterial hypertension*,‡
- Dysautonomia
- Cryoglobulinaemic vasculitis*

Pancreatic complications
- Recurrent acute pancreatitis

Nephro-urological complications
- Renal tubular acidosis (9% of patients)
- Glomerulonephritis (4% of patients)*
- Interstitial cystitis (in the absence of bacterial infection)
- Osteomalacia
- Recurrent renal colic due to renal stones
- Hypokalaemic paralysis*

Peripheral nervous system complications
- Mixed polyneuropathy
- Axon sensory polyneuropathy
- Sensory ataxic neuronopathy*
- Axon sensorimotor polyneuropathy
- Trigeminal or other cranial neuropathies
- Demyelinating polyradiculoneuropathy
- Autonomic neuropathy
- Pure sensory neuronopathy
- Mononeuritis multiplex*
- Small-fibre neuropathy (painful paresthesias)

Central nervous system complications
- White matter lesions (multiple sclerosis-like disease)*
- Neuromyelitis optica spectrum disorder*
- Recurrent aseptic meningitis

Haematological complications
- Haemolytic anaemia*,‡
- Unexplained leukopaenia (lymphopaenia and neutropaenia)
- Unexplained thrombocytopaenia*
- Evans syndrome‡
- Unexplained monoclonal gammopathy
- Thrombotic thrombocytopaenic purpura*,‡
- B cell lymphoma*

Obstetrics
- Autoimmune congenital heart block*
- Cardiac fibroelastosis
- Unexplained fetal valvular disease
- Neonatal lupus

Ear, nose and throat complications
- Recurrent parotid enlargement
- Bilateral multicystic parotid masses
- Sensorineural hearing loss
- Parotid lymphoma*

*Denotes severe systemic manifestations[74,84,90]. ‡Denotes rarely reported manifestations (<1% of patients) or suggesting polyautoimmunity (that is, the coexistence of other systemic autoimmune diseases).

Figure 1. Disease manifestations in primary Sjögren's syndrome
Nature Review Disease Primers 2016. Reprinted with permission

Epidemiology

pSS may develop in both sexes and in all ages, but women are significantly overrepresented with a female:male ratio of 14:1, and most women develop the disease during the post-menopausal period (12).

The estimated prevalence of the pSS varies, partly due to the different criteria and differences in study design used in the studies, but a fair estimation is a prevalence of 0.01–0.09 % (13-17). The incidence is estimated at 3.1/100 000 in a Swedish setting (18). There is an overlap between SS and other rheumatological diseases, i.e. one patient may have both rheumatoid arthritis, systemic lupus erythematosus (SLE), systemic sclerosis (SSc) or myositis and SS at the same time. When present together with another rheumatological disease, the condition is called secondary Sjögren's syndrome (sSS). The reported prevalence of sSS ranges between 1 and 18% (19, 20) amongst SLE patients, 7–17 % amongst RA patients (21, 22), and a prevalence of 12% is reported in patients with systemic sclerosis (SSc) (23). Due to the overlap in symptoms and findings it can sometimes be hard to categorise whether a patient has pSS with EGM or sSS. Also, different diagnostic traditions can affect which patient is given a clinical diagnosis of pSS.

Classification criteria

In order to overcome the diagnostic variations and to acquire homogeneous populations for scientific studies, different classification criteria sets for pSS have been developed. Several different criteria sets have been proposed e.g. the Copenhagen criteria (24), the Californian criteria (25), the Japanese criteria (26), the European criteria (27). Although the different criteria sets are similar, studies using different criteria sets can be hard to compare and thus efforts have been made to create international criteria sets. The first international criteria set was introduced in 1993 (27) and was updated in 2002 (the American-European consensus criteria (AECG)) (28), Table 2. In 2016 a new, simplified criteria set was established (the ACR/EULAR criteria) (29), Table 3. In this thesis, the AECG criteria are used in all studies. The AECG criteria and the ACR/EULAR criteria have been shown mostly to identify the same patients (30).

Table 1.
The American-European Consensus Group (AECG) criteria

1	**Ocular symptoms of dryness** – a positive response to at least one of the following questions: Have you had daily, persistent, troublesome dry eyes for more than 3 months? Do you have a recurrent sensation of sand or gravel in the eyes? Do you use tear substitutes more than three times a day?
2	**Oral symptoms of dryness** – a positive response to at least one of the following questions: Have you had a daily feeling of dry mouth for more than 3 months? Have you had recurrently or persistently swollen salivary glands as an adult? Do you frequently drink liquids to aid in swallowing dry food?
3	**Ocular signs** – objective evidence of ocular involvement determined on the basis of positive result in at least one of the following tests: Schirmer I test (abnormal if ≤ 5mm/5min) Van Bijsterveld score (abnormal if ≥ 4 points on a 0-9-point scale)
4	**Histopathology** – a focus score ≥ 1 in a minor salivary gland biopsy, with a focus defined as a conglomerate of more than 50 lymphocytes and a focus score defined as the number of foci per 4 mm^2 of glandular tissue.
5	**Salivary gland involvement** – objective evidence of salivary gland involvement determined on the basis of a positive result in at least one of the following three tests: Unstimulated whole sialometry Salivary gland scintigraphy Parotid sialography
6	**Autoantibodies** – presence in serum of the following antibodies: Antibodies to Ro (SS-A) or La (SS-B) or both.

Classification rules: For primary SS (pSS) – in patients without any potentially associated disease, pSS can be defined as follows: The presence of 4 of the 6 items is indicative of pSS as long as item 4 or 6 is positive. The presence of any of 3 of the 4 objective criteria items (3, 4, 5, 6).
For secondary SS (sSS) – in patients with a potentially associated disease (for instance another well-defined connective tissue disease), the presence of item 1 or item 2 plus any one from amongst items 3, 4 and 5 may be considered as indicative of sSS.
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 (for a period shorter than 4 times the half-life of the drug).

Table 2. The ACR/EULAR criteria

American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren's syndrome: The classification of primary Sjögren's syndrome (SS) applies to any individual who meets the inclusion criteria*, does not have any of the conditions listed as exclusion criteria,† and has a score of ≥4 when the weights from the five criteria items below are summed.

Item	Weight/score
Labial salivary gland with focal lymphocytic sialadenitis and focus score of ≥ 1 foci/4 mm2 ‡	3
Anti-SSA/Ro-positive	3
Ocular staining score (or van Bijsterveld score ≥ 4) in at least one eye §	1
Schirmer's test ≤ 5mm/5min in at least one eye §	1
Unstimulated whole saliva flow rate ≤ 0.1 ml/min**	1

*These inclusion criteria are applicable to any patient with at least one symptom of ocular or oral dryness, defined as a positive response to at least one 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 (SS) from the European League Against Rheumatism SS Disease Activity Index questionnaire (at least one domain with a positive item). †Exclusion criteria include prior diagnosis of any of the following conditions, which would exclude diagnosis of SS and participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests: (1) history of head and neck radiation treatment, (2) active hepatitis C infection (with confirmation by PCR), (3) AIDS, (4) sarcoidosis, (5) amyloidosis, (6) graft-versus-host disease, (7) IgG4-related disease. ‡The histopathologic examination should be performed by a pathologist with expertise in the diagnosis of focal lymphocytic sialadenitis and focus score count, using the protocol described by Daniels et al. §Patients who are normally taking anticholinergic drugs should be evaluated for objective signs of salivary hypofunction and ocular dryness after a sufficient interval without these medications in order for these components to be a valid measure of oral and ocular dryness. ¶Ocular Staining Score described by Whitcher et al; van Bijsterveld score described by van Bijsterveld. **Unstimulated whole saliva flow rate measurement described by Navazesh and Kumar.

Diagnostical work-up of pSS

The items included in the different classification criteria are similar and can be seen as a distillate of the clinical presentation of pSS. Some notes on the items in the AECG criteria are included below.

Dryness symptoms

Tears, saliva or other mucus produced by the exocrine glands are composite fluids including water, electrolytes, proteins and fat to achieve an effective lubricant as well as an antimicrobial effect (31, 32). Furthermore, saliva contains digestive enzymes (amylase, lipase) and growth factors (e.g. EGF) (32). When the glands are inflamed, not only the quantity of the secretion produced diminishes but also the quality of the secretion. Symptoms from the oral cavity and the eyes are the hallmark in pSS. Since mild or moderate symptoms of dryness, temporal or persistent, are common complaints in the general population, due to medication, different medical conditions, and age, the questions in the criteria focus on longstanding, severe problems.

Objective measurements of lacrimal gland function

Several different methods exist for evaluation of impaired lacrimal gland function (33) and some are presented below.

- Measurement of tear volume: Schirmer's test, which measures the amount of tears produced, is the most widely used measurement of lacrimal gland function. A strip of filter paper is applied to the lower conjunctival sack and the wetting length is measured after 5 minutes. The cut-point is set at ≤ 5mm for a positive result.
- Evaluation of surface damage: Dryness of the eyes causes damage to the corneal and conjunctival surface. The damaged epithelia can be dyed with Rose-Bengal or Lissamine green and scored. The most used scoring system is the van Bijsterveld score (34). More recently, another scoring system has been developed, the ocular staining score (OSS) (35).
- Evaluation of tear-stability: Due to both changed composition and volume of tears, the time before the tear film breaks up on the corneal surface is diminished in pSS. Tear break-up time (BUT) can be used as a measurement of both tear quality and quantity, but is not used in the current criteria sets.
- Measurement of osmolarity: The osmolarity of the tear film can be measured, since inflammation alters the osmolarity. This is rarely used in clinical practice and is not included in current criteria sets.

Objective measurement of salivary gland function

As for the evaluation of lacrimal glands, several different methods exist for evaluation of the salivary glands (36).

- Unstimulated whole saliva measurement (UWS) is the method most commonly used to assess the function of the salivary glands. The patient leans over and lets all produced saliva drip into a container during 15 minutes. A result ≤ 1.5 ml is considered pathologic.
- Parotid gland scintigraphy is a nowadays a rarely used method assessing salivary gland function.
- Parotid gland sialography is a nowadays rarely used method due to its invasive character.
- MR for salivary glands has been evaluated but is rarely used due to cost and limited access.
- Ultrasound is an interesting alternative since it is non-invasive and easily accessible but requires training of the assessor.

Salivary gland histopathology

Autoimmune inflammation in the salivary glands, so called focal sialoadenitis, is characteristic for pSS. Focal sialoadenitis is defined as a presence of ≥ 1 foci of lymphocytes (≥ 50 lymphocytes), aggregated around the salivary ducts, per $4mm^2$

of glandular tissue. This corresponds to grade 3–4 in the previously used Chisholm-Mason grading system (37).

A focus score of ≥ 1 is shown to be associated to low unstimulated but not low stimulated whole salivary flow rates (36). Of note, a lip biopsy with a focus score of ≥ 1 is not 100 % specific for pSS, as studies have found a focus score of ≥ 1 in up to 23% of healthy controls (38, 39). Also, lip biopsies from patients suffering from other autoimmune diseases such as rheumatoid arthritis, mixed connective tissue disease (MCTD), ankylosing spondylitis and myasthenia gravis may also show a positive result (40, 41).

In pSS the population of inflammatory cells changes as the lesions progress: the number of total T-cell, CD4+ T-cells, T/B-cell ratio and iDC are negatively correlated and B-cells and macrophages are positively correlated to the grade of inflammation (42). The infiltration of macrophages is also associated with a more severe disease and presence of germinal centres in the biopsies has also been shown to be a predictor of lymphoma development (43).

Serological findings

Many different autoantibodies are found in pSS patients, the most specific autoantibodies for pSS being the Sjögren's syndrome A (SSA)- and Sjögren's syndrome B (SSB) antibodies, also named anti-Ro and anti-La antibodies, respectively. Anti-Ro/SSA and anti-La/SSB were originally described as two precipitation antibodies in patients with pSS in 1961 (44). Anti Ro/SSA and anti La/SSB is found in 73% and 45% of pSS patients respectively (45). Anti Ro/SSA is also found in other diseases such as SLE, SSc, PBC, RA, and myositis (46). In contrast, anti-SSB/La is more closely associated to pSS and SLE (47). In pSS, the presence of anti-SSA and anti-SSB in serum is associated with activation of the immune system (the so-called type I interferon (IFN) signature), increased expression of B-cell activating factor (BAFF) as well as with the development of EGM (45, 48, 49). Other autoantibodies frequently found in pSS include anti-nuclear antibodies (ANA) and rheumatoid factor (RF), which are found in 79% and 49% of pSS patients respectively (45). RF is an antibody binding the Fc part of IgG and can be of any isotype e.g. IgG, IgA or IgM type. In clinical practice, IgM-RF is most commonly measured. Importantly, RF can cause interference in sandwich ELISA assays by binding to the detecting antibodies in the assay (50).

Ro/SSA

The Ro/SSA antigen consist of two cellular proteins (Ro 60 and Ro52) which were first believed to belong to the same complex. Later studies suggest that Ro 52 (localized in the cytoplasm) and Ro 60 (localized in the nucleus and nucleolus) were distinct. The cause of the association between presence of anti-Ro52 and anti-Ro60 is unknown.

Ro60: The function of Ro60 in unknown, although it has been shown to bind small non-coding RNAs termed Y-RNAs. The crystal structure of Ro60 suggests that it can bind both single stranded and double stranded RNA and may function as a chaperon binding misfolded RNA targeting it for degradation (51). Ro60 may also bind viral RNA. Knock-out mice for Ro60 develop autoimmune disease with anti-chromatin and anti-ribosome antibodies and glomerulonephritis i.e. a disease similar to SLE (52). One epitope on the Ro60 antigen cross-reacts with an epitope on EBV (EBNA-1) supporting the theory that EBV may have a triggering role in the development of anti-Ro60 autoantibodies (53).

Ro52: Ro52 is interferon inducible and is induced by viral infection or stimulation of Toll-like receptors (TLR). Ro52 is an E3-ubiquitin ligase which adds a ubiquitin to its target. Ro52 exerts an anti-inflammatory effect as it is shown to downregulate IRF3 and 7 and IKKβ (54-56). Furthermore, Ro52 knock-out mice develop uncontrolled inflammation after minor injury (57) and allelic polymorphisms in the Ro52 gene are associated with Ro52 autoantibodies in pSS (58).

The risk of foetal heart block is associated with high titers of anti-Ro60 and/or anti-Ro52 autoantibodies in the pregnant mother, and is due to expression of Ro antigen in foetal cardiac myocytes during the 18–24 week of gestation. The Ro52 subtype p200 has been highlighted as being of special interest for its connection to foetal heart block (59).

The clinical and pathological role of SSA is further reviewed by Yoshimi (46).

La/SSB

La/SSB is a 47 kDa protein is involved in the process of small, non-coding RNA and travels between the nucleus and the cytoplasm. Patients positive for anti-SSB are with few exceptions also positive for SSA (45). Mice immunised with recombinant SSB also develop antibodies to Ro60 and reciprocally, mice immunised with Ro60 also develop anti-SSB antibodies (60). This suggests that the initial response to an epitope is perpetuated and via epitope spreading causes development of several autoantibodies.

ESSDAI and ESSPRI

To enable a uniform assessment of the activity of the disease and the burden of symptoms, two indices have been developed. The EULAR Sjögren's syndrome Activity Index (ESSDAI) measures disease activity in twelve different domains where different organ systems are assessed as well as laboratory analyses mirroring disease activity (Table 3). The total score is calculated by adding the individual domains scores, which gives a total score ranging between 0 and 123. Low, moderate and high disease activity are defined as a score of 0–4, 5–13 and ≥14, respectively. The ESSDAI is constructed to assess active, potentially treatable,

inflammation rather than (irreversible) damage. The threshold for clinically meaningful improvement is set at ≥ 3 points (61-64). Approximately, one third of pSS patients have EGMs and thus the ESSDAI is of limited use in clinical studies for two-thirds of patients. Additionally, not all domains of ESSDAI are sensitive to change. Most frequent changes are seen in the biological, haematological, pulmonary, articular and glandular domains (65).

In the ESSPRI, patients score symptoms experienced the last 2 weeks in three different domains: dryness, pain, and fatigue on a Likert scale between 0 and 10 (Figure 2). The total ESSPRI score is calculated as the mean of the different domains and thus range between 0 and 10. The threshold for clinically meaningful improvement in ESSPRI is set at 1 point or 15% improvement (62, 66).

Table 3.
EULAR Sjögren's syndrome disease activity index. Domain items and weights

Domain [Weight]	Activity	Description
Constitutional [3] *Exclusion of fever of infectious origin and voluntary weight loss*	No = 0	Absence of the following symptoms
	Low = 1	Mild or intermittent fever (37.5°–38.5°C)/night sweats and/or involuntary weight loss of 5 to 10% of body weight
	Moderate = 2	Severe fever (>38.5°C)/night sweats and/or involuntary weight loss of >10% of body weight
Lymphadenopathy [4] *Exclusion of infection*	No = 0	Absence of the following features
	Low = 1	Lymphadenopathy ≥ 1 cm in any nodal region or ≥ 2 cm in inguinal region
	Moderate =2	Lymphadenopathy ≥ 2 cm in any nodal region or ≥ 3 cm in inguinal region, and/or splenomegaly (clinically palpable or assessed by imaging)
	High = 3	Current malignant B-cell proliferative disorder*
Glandular [2] *Exclusion of stone or infection*	No = 0	Absence of glandular swelling
	Low =1	Small glandular swelling with enlarged parotid (≤ 3 cm), or limited submandibular or lachrymal swelling
	Moderate =2	Major glandular swelling with enlarged parotid (> 3 cm), or important submandibular or lachrymal swelling
Articular [2] *Exclusion of osteoarthritis*	No = 0	Absence of currently active articular involvement
	Low = 1	Arthralgias in hands, wrists, ankles and feet accompanied by morning stiffness (>30 min)
	Moderate =2	1 to 5 (of 28 total count) synovitis
	High = 3	≥ 6 (of 28 total count) synovitis
Cutaneous [3] *Rate as "No activity" stable long-lasting features related to damage*	No = 0	Absence of currently active cutaneous involvement
	Low =1	Erythema multiforma
	Moderate = 2	Limited cutaneous vasculitis, including urticarial vasculitis, or purpura limited to feet and ankle, or subacute cutaneous lupus
	High = 3	Diffuse cutaneous vasculitis, including urticarial vasculitis, or diffuse purpura, or ulcers related to vasculitis
Pulmonary [5] *Rate as "No activity" stable long-lasting features related to damage, or respiratory involvement not related to the disease (tobacco use etc.)*	No =0	Absence of currently active pulmonary involvement
	Low = 1	Persistent cough or bronchial involvement with no radiographic abnormalities on radiography Or radiological or HRCT evidence of interstitial lung disease with: No breathlessness and normal lung function test.
	Moderate = 2	Moderately active pulmonary involvement, such as interstitial lung disease shown by HRCT with shortness of breath on exercise (NHYA II) or abnormal lung function tests restricted to: 70% >DLCO ≥ 40% or 80%>FVC≥60%
	High = 3	Highly active pulmonary involvement, such as interstitial lung disease shown by HRCT with shortness of breath at rest (NHYA III, IV) or with abnormal lung function tests: DLCO < 40% or FVC< 60%
Renal [5] *Rate as "No activity" stable long-lasting features related to damage, and renal involvement not related to the disease.* *If biopsy has been performed, please rate activity based on histological features first*	No = 0	Absence of currently active renal involvement with proteinuria< 0.5 g/d, no haematuria, no leukocyturia, no acidosis, or long-lasting stable proteinuria due to damage
	Low = 1	Evidence of mild active renal involvement, limited to tubular acidosis without renal failure or glomerular involvement with proteinuria (between 0.5 and 1 g/d) and without haematuria or renal failure (GFR ≥60 ml/min)
	Moderate = 2	Moderately active renal involvement, such as tubular acidosis with renal failure (GFR <60 ml/min) or glomerular involvement with proteinuria between 1 and 1.5 g/d and without haematuria or renal failure (GFR ≥60 ml/min) or histological evidence of extra-membranous glomerulonephritis or important interstitial lymphoid infiltrate
	High = 3	Highly active renal involvement, such as glomerular involvement with proteinuria >1.5 g/d or haematuria or renal failure (GFR <60 ml/min), or histological evidence of proliferative glomerulonephritis or cryoglobulinemia related renal involvement

Muscular [6] *Exclusion of weakness due to corticosteroids*	No = 0	Absence of currently active muscular involvement
	Low = 1	Mild active myositis shown by abnormal EMG or biopsy with no weakness and creatine kinase (N <CK ≤ 2N)
	Moderate =2	Moderately active myositis proven by abnormal EMG or biopsy with weakness (maximal deficit of 4/5), or elevated creatine kinase (2N<CK ≤4N)
	High = 3	Highly active myositis shown by abnormal EMG or biopsy with weakness (deficit ≤ 3/5) or elevated creatine kinase (>4N)
PNS [5] *Rate as "No activity" stable long-lasting features related to damage or PNS involvement not related to the disease*	No = 0	Absence of currently active PNS involvement
	Low = 1	Mild active peripheral nervous system involvement, such as pure sensory axonal polyneuropathy shown by NCS or trigeminal (V) neuralgia
	Moderate = 2	Moderately active peripheral nervous system involvement shown by NCS, such as axonal sensory-motor neuropathy with maximal motor deficit of 4/5, pure sensory neuropathy with presence of cryoglobulinaemic vasculitis, ganglionopathy with symptoms restricted to mild/moderate ataxia, inflammatory demyelinating polyneuropathy (CIDP) with mild functional impairment (maximal motor deficit of 4/5or mild ataxia), Or cranial nerve involvement of peripheral origin (except trigeminal (V) neuralgia)
	High = 3	Highly active PNS involvement shown by NCS, such as axonal sensory-motor neuropathy with motor deficit ≤3/5, peripheral nerve involvement due to vasculitis (mononeuritis multiplex etc.), severe ataxia due to ganglionopathy, inflammatory demyelinating polyneuropathy (CIDP) with severe functional impairment: motor deficit ≤3/5 or severe ataxia
CNS [5] *Rate as "No activity" stable long-lasting features related to damage or CNS involvement not related to the disease*	No = 0	Absence of currently active CNS involvement
	Low = 1	Moderately active CNS features, such as cranial nerve involvement of central origin, optic neuritis or multiple sclerosis-like syndrome with symptoms restricted to pure sensory impairment or proven cognitive impairment
	High = 3	Highly active CNS features, such as cerebral vasculitis with cerebrovascular accident or transient ischemic attack, seizures, transverse myelitis, lymphocytic meningitis, multiple sclerosis-like syndrome with motor deficit.
Haematological [2] *For anaemia, neutropenia, and thrombopenia, only auto-immune cytopenia must be considered. Exclusion of vitamin or iron deficiency, drug-induced cytopenia*	No = 0	Absence of auto-immune cytopenia
	Low = 1	Cytopenia of auto-immune origin with neutropenia (1000 < neutrophils < 1500/mm3), and/or anaemia (10 < haemoglobin < 12 g/dl), and/or thrombocytopenia (100,000 < platelets < 150,000/mm3) or lymphopenia (500 < lymphocytes < 1000/mm3)
	Moderate = 2	Cytopenia of auto-immune origin with neutropenia (500 ≤ neutrophils ≤ 1000/mm3), and/or anaemia (8 ≤ haemoglobin ≤ 10 g/dl), and/or thrombocytopenia (50,000 ≤ platelets ≤ 100,000/mm3) or lymphopenia (≤500/mm3)
	High = 3	Cytopenia of auto-immune origin with neutropenia (neutrophils < 500/mm3), and/or or anaemia (haemoglobin < 8 g/dl) and/or thrombocytopenia (platelets <50,000/mm3)
Biological [1]	No = 0	Absence of any of the following biological feature
	Low = 1	Clonal component and/or hypocomplementemia (low C4 or C3 or CH50) and/or hypergammaglobulinemia or high IgG level between 16 and 20 g/L
	Moderate = 2	Presence of cryoglobulinemia and/or hypergammaglobulinemia or high IgG level > 20 g/L, and/or recent onset hypogammaglobulinemia or recent decrease of IgG level (<5 g/L)

*Defined as indolent not treated lymphoma or currently treated lymphoma or myeloma (or treatment ended from less than 6 months). Do not rate past treated lymphoma or myeloma in complete remission. CIDP= chronic inflammatory demyelinating polyneuropathy; CK= creatine kinase; CNS= central nervous system; DLCO= diffusing CO capacity; EMG= electromyogram; FVC= forced vital capacity; GFR= glomerular filtration rate; Hb= haemoglobin; HRCT= high-resolution computed tomography; IgG= immunoglobulin G; NCS= nerve conduction studies; NHYA= New York heart association classification; Plt= platelet; PNS=peripheral nervous system;

1) How severe has your dryness been during the last 2 weeks ?

No dryness	0	1	2	3	4	5	6	7	8	9	10	Maximal imaginable dryness

2) How severe has your fatigue been during the last 2 weeks ?

No fatigue	0	1	2	3	4	5	6	7	8	9	10	Maximal imaginable fatigue

3) How severe has your pain (joint or muscular pains in your arms or legs) been during the last 2 weeks ?

No pain	0	1	2	3	4	5	6	7	8	9	10	Maximal imaginable pain

Figure 2.
The EULAR Sjögren's syndrome Patient Reported Index (ESSPRI)

Pathogenesis

What causes the development of pSS is not fully understood. In the current understanding, there is an interplay between intrinsic factors (e.g. genetics and sex hormones) and extrinsic factors (e.g. virus infections). In a susceptible individual, mucosal epithelial cells may be activated, possibly by viral infection or activation or by endogenous viral products, resulting in an activation of both the innate and adaptive immune system with the subsequent production of autoantibodies. The antibodies form immune complexes with apoptotic material that maintain and augment the inflammatory response, creating a vicious circle leading to tissue damage. Clues to what contributes to susceptibility to developing pSS, possible triggers and the immunological response are presented below.

Epidemiological risk factors

Sex

Studies on epidemiological risk factors, other than the impact of sex, for the development and course of the disease are few. As mentioned above, women are primarily affected (67) and most cases are diagnosed in their middle age (68). The

reports of men having pSS is conflicting: some report a lower prevalence of immunological findings whilst others report a higher prevalence (69, 70).

Studies comparing systemic manifestations of pSS between men and women report a higher prevalence lymphoma and pulmonary manifestations amongst men and a higher prevalence of Raynaud's phenomenon, thyroiditis and fibromyalgia amongst women (69, 70). There are differences in the prevalence of several autoimmune diseases depending on sex, and women are commonly overrepresented (71). In several rheumatological diseases, periods of changes in sex hormone levels amongst women such as during menarche, menopause, pregnancy and the period after delivery are all critical periods for developing disease (72). Notably, the most common period for women to develop pSS is around the menopause, which indicates that a reduction in oestrogen concentration might influence the development of the disease.

Ethnicity

Few studies have investigated the effect of ethnicity on the prevalence of pSS. One study, from the population of Greater Paris, found that non-Europeans had a two-fold higher risk of pSS, were younger at diagnosis, and had higher rates of hypergammaglobulinaemia and anti-SSA compared to Europeans (15). Another study from Manhattan did not find any difference between different ethnic groups (73). A multicentre, international study entailing over 8,000 patients, which investigated the effect of ethnicity on the clinical presentation of the disease, found that the female-to-male ratio was highest amongst Asian patients (27:1) and lowest amongst black/African-American patients (7:1). A higher frequency of positive salivary gland biopsies was found amongst white and Hispanic patients. The study also found that the patients in the northern countries in Europe had the lowest prevalence of ANA seropositivity. In addition, a north-south gradient was also found in respect to the prevalence of dry eyes and abnormal ocular tests (12).

Smoking

Several studies have found a lower prevalence of current smokers amongst pSS patients (74-79). In one study, smoking data from a health survey, performed years before the diagnosis of pSS, was analysed. In the group of individuals, later diagnosed with pSS, there was a lower prevalence of current smokers compared to controls and a higher prevalence of former smokers (79). A common explanation for the lower frequency of smokers is that smoking causes irritation of the dry mucosal surfaces in the eyes, oral cavity and airways, making pSS patients more prone to give up smoking. Although this certainly has an impact on the willingness to quit, it does not quite explain the findings on smoking habit years before diagnosis. Interestingly, a large study from the United States, using non-pSS sicca patients as controls, also found a lower frequency of smoking in pSS patients

compared to controls (80). This study also found a lower frequency of focal sialadenitis and SSA antibodies in current smoking pSS patients, reminiscent of the findings in a previous study from Sweden (81), indicating that smoking might have an impact on the development of the disease. Studies comparing smoking rates between pSS patients and controls are presented in Table 4. The negative effects of cigarette smoking are well known and include the increased risk of several forms of cancer (82, 83), cardiovascular disease (84) chronic obstructive pulmonary disease (COPD) (85) and autoimmune diseases such as RA (86) and Crohn's disease (87). On the other hand, there are also diseases where smoking may exert beneficial effects such as ulcerative colitis (87), Behcet's disease (88) and Parkinson's disease (89). Cigarette smoke contains several thousand of different chemical compounds (90) and is shown to have both proinflammatory and anti-inflammatory effects (91, 92). One reason, both for the increased risk of cancer and the effect in inflammatory diseases, might be a direct effect on the immune system (92). Smoking is also shown to downregulate the effect of type I IFNs via downregulation of it's receptor (93) which is of particular interest since the IFN system is crucial in the pathogenesis of pSS.

Table 4.
Prevalence of smoking in pSS patients and controls in studies including smoking habits in pSS

Authors (ref). Country	Year	Smoking status	pSS vs controls	P-value (fraction smokers pSS vs controls)	N pSS/N controls	Criteria
Manthorpe (81) Sweden	2000	Current	24 vs 28%	0.14	355/3700	Copenhagen criteria
		Former	25 vs 22%			
		Never	51% vs 50%			
Priori (74) Italy	2007	Current	15.1 vs 23.2%	OR 0.8 (0.4-1.6)	140/109	AECG
		Former	17.3 vs 12.0%	OR 1.5 (0.7-3.1)		
		Never	67.6 vs 64.8%	OR 1		
Karabulut (75) Turkey	2011	Current	11.6 vs 22.3%	0.0012	207/602	AECG.
		Former	30.4 vs 11.8%	<0.0001		
		Never	58.0 vs 65.9%	<0.0001		
Stone (80) USA	2017	Current	4.6 vs 14.1%	<0.0001	587/701	AECG. pSS vs non pSS sicca patients
		Former	30.5 vs 32.8%	0.37		
		Never	64.9 vs 53.1 %	0.0003		
Olsson (79) Sweden	2017	Current	17 vs 37%	0.003	63/252	AECG. Pre-diagnostic data.
		Former	53 vs 25%	<0.001		
		Never	30 vs 38%	0.23		
Vaudo (94) Italy	2005	Current	21 vs 17%	0.75	37/35	AECG
		Former	24 vs 20%	0.74		
Pérez-De-Lis (77) Spain	2010	Ever	19 vs 31%	<0.001	312/312	1993 classification criteria.
Gerli (95) Italy	2010	Current	18 vs 17%	0.29	45/59	AECG
Juarez (76) UK	2014	Current	3.8 vs 10.1 %	0.026	200/200	AEGC.
Bartoloni (78) Italy	2015	Ever	13 vs 23%	<0.001	788/4774	AECG
Atzeni (96) Italy	2014	Current	0 vs 0		22/22	ACR-criteria
Sabio (97) Spain	2015	Current	23 vs 37	0.11	44/78	AECG.
Balarini (98) Brazil	2016	Current?	3 vs 3	0.89	63/63	AECG
Zardi (99) Italy	2014	Current?	17 vs 17	>0.99	18/18	AECG/ACR
Zardi (100) Italy	2016	Current	0 vs 0	>0.99	25/22	AECG
Augusto (101) Brazil	2016	Current	4.2 vs 2.8	1.0	71/71	AECG.
		Former	18.3 vs 23.9	0.54		

Miscellaneous

High birth weight has also been reported as a risk factor for developing pSS later in life (102). The reason for the association is not known. Furthermore, in contrast to studies of RA diseases (103, 104), it has not been shown that socioeconomic status or educational level have an impact on the development of pSS (74, 79). Finally, exposure of organic solvents has been associated with pSS (105).

Genetics

Genealogy

The genealogic mapping in pSS is poorly studied, but there are reports of familial clustering and twins having pSS (106-108). A recent study found that having a twin sibling with pSS is associated with a 662-fold increased risk, having a sibling a 19-fold increased risk, and being the offspring of a pSS patient an 11-fold increased risk of being diagnosed with pSS (109). The study also showed an increased risk of being diagnosed with other autoimmune diseases in patients with a first-degree relative suffering from pSS. A similar clustering has been described by others (110).

Genetic studies

Since pSS predominantly affects females, the X chromosome has been of special interest in genetic studies. Studies have shown that Klinefelter's syndrome (47, XXY) is associated with an increased risk of being diagnosed with pSS (111). Furthermore, Turner's syndrome (45, X) is rarely associated with pSS, whilst women with 47, XXX have an increased risk of pSS (112), suggesting that the X-chromosome carries genes increasing the risk of pSS.

The strongest genetic associations found so far are related to the HLA class II region: HLA-DQA1*0501, HLA-DQB1*0201 and HLA- DRB*0301 (113). These are molecules presenting extracellular antigens to the immune system and variants may be more or less prone to present autoantigens or to supporting epitope spreading. Studies have shown that the HLA II gene variants are associated with the presence of anti-SSA and anti-SSB antibodies, although no correlation was found to disease severity (114). Interestingly, EBV uses HLA class II for entering B-cells (115) which might have importance for disease development. Furthermore, GWAS (Genome Wide Association Studies) have revealed associations genes involved in B-cell activation (EBF-1, BLK, TNFSF4), IFN I and II signalling (IRF5, IL12A, STAT4, OAS1), NF-κB signalling (TNIP1 and TNFAIP3) and lymphocyte trafficking (CXCR5) (113, 116, 117). The associations are interesting, but how these polymorphisms affect the biologic function is not yet unravelled.

Epigenetics

Epigenetic changes comprise mitotically heritable changes that can potentially influence the phenotype without involving any alteration to the DNA sequence itself. The main epigenetic regulation is accomplished via DNA methylation, histone modifications and micro (mi) RNA.

Post-translational modifications of histone proteins and DNA methylation affect gene expression by altering the accessibility of the DNA to DNA transcriptase. Mi-RNA can bind to RNA and interfere with its translation to proteins.

In pSS, three epi-genome-wide association studies (EWASs) on peripheral blood cells have shown hypomethylation at IFN-induced genes in different cell types. The hypomethylations was also found to be more pronounced in patients with anti-SSA and/or anti-SSB antibodies (118-120). A study of cultured salivary gland cells from pSS patients and controls also showed a different methylation of IFN-induced genes amongst pSS patients (121) which supports the role of the salivary glands in the sustaining of inflammation (122).

Hormones

Sex hormones, especially oestrogen, has been shown to affect both the innate and the adaptive immune system (71). During pregnancy, when oestrogen and progesterone levels are high, the immune system must be adjusted in such a way that it does not attack the foetus, promotes survival of the foetus and does not jeopardize the survival of the mother. It is well known that the immune system skews from Th1 to Th2 during pregnancy, which is adequate since the immunoglobulins is transferred to the foetus and protects the child from infections during the first months after delivery (123). It is also well known that certain rheumatological diseases such as RA are ameliorated during pregnancy whilst others, such as SLE, can be worsened, which reflects differences in pathogenesis (71). Mice models have shown that oestrogen suppresses the development of pSS in normal mice and that ovariectomy leads to a condition similar to pSS (124). It is also shown that oestrogen can inhibit T-cell recruitment in salivary glands (125) and prevent cell death in lacrimal glands (126). Additionally, in mice models, administration of oestrogen prevented development of sialoadenitis and mice lacking aromatase, which converts androgens to oestrogens, develop lymphocytic exocrinopathy similar to pSS (127). Androgens have also been shown to decrease lymphocytic infiltration in salivary glands and low levels of DHEA, both systemically and in salivary glands in pSS patients, have been demonstrated (128). Cigarette smoking is associated with higher levels of androgens and oestrogen amongst postmenopausal women (129), which is of interest, since smoking has been shown to be negatively associated to pSS. Sex hormones also affect the function of

exocrine glands. Hormone replacement therapy (HRT), including oestrogen and progesterone, is shown to increase salivary flow, buffer effect and pH when given as hormone replacement therapy to peri- and postmenopausal women (130). On the other hand, HRT is also associated with a higher frequency of dry eye syndrome, affecting both lacrimal and Meibomian glands (131).

Viruses

The immunological response causing pSS has a great similarity with the response to viral infections, namely stimulation of TLR, type I IFN production, production of immune complexes, and B-cell hyperactivity. The role of viral infections as a cause of pSS, initiating the response later developing into the disease (via epitope spreading) or sustaining the disease has been under investigation for decades (132). Considering the female overrepresentation amongst pSS patients, and that the inflammation involved in pSS is similar to the response to viruses, it is interesting that women generally has a stronger response to viral infections (133). In addition, many viruses can infect and reside in salivary glands (so-called sialotropic viruses) which can be an advantage for virus transmission. Examples of sialotropic viruses are EBV, CMV, and Hepatitis C virus.

The virus that has caught most attention is the Epstein Barr virus (EBV) also called human herpes virus 4 (HHV-4).

Epstein Barr virus (HHV-4)

It is estimated that over 90% of the world population is infected with EBV. Often, EBV is transmitted by saliva during childhood and may then result in a subclinical infection. If the infection takes place later in life, it can cause infectious mononucleosis ("kissing disease") with lymphadenopathy, lengthy fever and malaise. EBV is, as for other viruses from the herpes group, a dsDNA virus. EBV primarily infects B-cell although several different cells types can be infected including, epithelial cells, T-cells and myocytes. When EBV infects a naïve B-blast, two EBV genes (LMP-1 and LMP-2) allow the cell to transform to a resting memory B-cell and thus become a virus reservoir. Epithelial cells in the orophanynx are thought be infected by EBV from B-cells in a cyclical manner, amplifying the shedding of EBV (134). In the vast majority, the infection does not cause disease as it is controlled by the immune system. However, EBV is also linked to the development of lymphoma, especially Burkitt, Hodgkin, and diffuse large B-cell lymphomas (135). In vitro, EBV infected B-cells will grow indefinitely, and the proliferative effect by the EBV infection is thought to be the reason for the increased risk of lymphoma. Individuals suffering from immunosuppression, caused by infections (e.g. infection by human immunodeficiency virus (HIV) or *Plasmodium falciparum*) or drug treatment, have increased risk of EBV driven malignancies due

to the impaired immunosurveillance by T-cells (135). Interestingly, EBV infected cells reside and participate in the germinal centres although it is not clear whether EBV exploit the GC's or actively generate a GC-like environment (136).

EBV and pSS

EBV DNA has been found in higher frequencies in salivary gland biopsies from pSS patients compared to controls (137, 138). Furthermore, active EBV infection in salivary glands has been associated with ectopic lymphoid structures, which is not surprising considering previous reports that EBV resides in GCs. Interestingly, perifollicular plasma cells infected by EBV frequently displayed Ro52 reactivity in salivary gland biopsies from pSS patients, whereas plasma cells from RA synovia showed reactivity to citrullinated peptides (139), suggesting that EBV infection might facilitate autoimmunity. Furthermore, an increased production of EBV by B-lymphocytes derived from pSS patients has been found (140), saliva from pSS patients has been shown to activate EBV in vitro, (141), increased levels of EBV antibodies in sera from pSS has been demonstrated (142, 143), and one epitope on Ro60 cross-reacts with EBNA-1 (53) which taken together point to a possible role of EBV in the pSS pathogenesis.

Although there is an association between pSS and EBV, it is not clear whether EBV causes the autoimmune response in the disease or that pSS results in a reactivation of EBV infection (144).

Other viruses that have been implicated in pSS pathogenesis include hepatitis C-virus (145), human T-lymphocyte virus type 1 (HTLV-1) (146) and coxsackie virus (147).

Since many viruses have been associated with pSS, it might well be that *a* viral infection rather than *a specific* viral infection is important for the development of pSS.

Cytokines

Cytokines are a large group of small secreted proteins used for communication between cells, especially leukocytes. Factors influencing the biological response of a cytokine include which cell type is stimulated, the concentration of the cytokine, the concentration of other cytokines, and the signals coming from other receptors (e.g. the T-cell receptor) which makes the mapping of these pathways complex. An inflammatory process always includes many different cell types and a plethora of cytokines. That said, certain cytokines are shown to be more important in different immunological responses (e.g. against viruses, bacteria, parasites or autoimmune processes) and despite the complexity, the inhibition of one cytokine (e.g. TNFα or IL-6) can be sufficient to inhibit an inflammatory process, e.g. in rheumatoid

arthritis. In pSS type I interferons (IFN) and B-cell activating factor (BAFF) have caught special attention.

Type I interferons

The importance of type I interferons (IFN) in the pathogenesis of pSS is well established. Type I IFNs is a group of related cytokines signalling through the same receptor (IFNAR). The term "interferon" comes from the initial observation that type I IFNs interfered with virus replication (148). Type I IFNs include IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. IFN-α is further divided into 13 subtypes (149). Although most cells can produce smaller amounts of type I IFNs, the main producer is the plasmacytoid dendritic cell (pDC) (150). The production of type I IFN is typically induced by viruses, bacteria, or microbial nucleic acids when sensed by pattern recognition receptors (PRRs). PRRs include toll-like receptors (TLRs) on endosomal membranes, retinoic acid inducible gene 1 (RIG-1)-like receptors (RLRs), and DNA sensing receptors (DSR) in the cytoplasm (151). pDCs express TLR-7 (sensing single stranded RNA) and TLR-9 (sensing microbial double-stranded DNA) on their endosomal membranes and can thus be activated when infected via receptor-mediated endocytosis. Furthermore, immune complexes containing DNA or RNA can activate pDC when recognized by FcγRIIa receptors (150). Thousands of genes are activated/affected when type I IFNs bind to its receptor (152) and the consequences include pro-apoptotic and anti-proliferative effects and production of proteins inhibiting viral transcription and promoting viral RNA degradation (153). During apoptosis, autoantigens are released that potentially promote autoimmune inflammation partly by an increased production of proteins serving as autoantigens e.g. Ro52 (154). Type I IFNs also activate dendritic cells (DCs), macrophages, B-cells and NK-cells (155).

The most common way to measure a cytokine concentration in serum or other bodily fluids to date, is by enzyme-linked immunosorbent assay (ELISA), which, however, is suboptimal when assessing activity in the type I IFN system due to the number of IFNs stimulating the same receptor. Instead, a common approach is to the measure the transcription of a selection of type I IFN stimulated genes by reverse transcriptase polymerase chain reaction (RT-PCR) (156).

In pSS patients, increased expression of IFN related genes is found systemically in approximately 60% of pSS patients (157, 158) and type I IFN producing cells, such as pDCs, are found in salivary gland tissue (158-160). Additionally, sera from pSS patients stimulates type I IFN production in normal peripheral blood mononuclear cells when combined with apoptotic or necrotic material (159) indicating that a disturbed clearance of apoptotic cells and the formation of immune complexes are involved in the pathogenesis. Furthermore, smoking is shown to attenuate type I IFN signalling by down regulation of the IFN receptor (93) which is of interest, since pSS has been shown to be negatively associated with smoking.

To add to the complexity, paradoxically, low dose IFN-α has also been evaluated as a treatment for pSS in a study showing an increased tear and salivary flow in IFN-α treated pSS patients. Additionally, 9 responders in the study that were re-biopsied showed ameliorated lymphocytic infiltration in the glands after treatment (161). Finally, low dose IFN-α has been shown to increase the expression of aquaporin 5, an important water channel protein in exocrine glands, which could possibly explain the improvement of salivary flow in IFN-α treated pSS patients (162).

B-cell activating factor (BAFF)

Augmented production of immunoglobulins (IgG, IgA and IgM) is a common feature in pSS. As mentioned earlier, pSS patients also have high rates of autoantibodies, which indicates an overactivation of B-lymphocytes. Infiltrating B-lymphocytes are also found in salivary gland biopsies (42). BAFF is a cytokine crucial for the maturation and survival of B-lymphocytes and augmented concentrations of this cytokine is found in pSS patients (163). The production of BAFF is increased by type I IFNs (164), and levels of BAFF correlate to titers of autoantibodies in pSS patients (165). Furthermore, BAFF-transgenic mice develop manifestations similar to pSS (166, 167). Therefore, antibodies targeting BAFF, that are currently used for the treatment of SLE (168), have also been studied in pSS (169).

Other cytokines

Apart from type I IFN and BAFF, many other cytokines are shown to be aberrantly expressed in pSS. These include cytokines produced by Th1 cells (IL-2, IFN-γ), TH2 cells (IL-4, IL-5, IL-13), Th-17 cells (IL-17) and generally proinflammatory cytokines (TNF-α, IL-1β, IL-6), some of which are presented briefly below:

TNFα is produced mainly by macrophages and T-cells and stimulates the acute phase reaction in the liver and activates macrophages. Increased levels of TNF-α have been found in both serum (170), saliva (171) and salivary gland biopsies (172) in pSS patients.

IL-6 has pleiotropic effects and its production is stimulated by TNF-α. Effects include the initiation of the complete acute phase reaction in the liver, stimulation of antibody production as well as the downregulation of TNF-α. Several studies have shown elevated levels of IL-6 in pSS (173-175).

IL-10 is mainly an anti-inflammatory cytokine but can act pro-inflammatory in certain conditions (176). Increased levels of IL-10 has been found in saliva from pSS patients (177), increased production of IL-10 by peripheral blood mononuclear cells from pSS patients (178) and genetic polymorphisms of the promotor of the IL-10 gene are associated with pSS (179).

IL-12 stimulates T-cells and NK cells and the production of IFN-γ and is shown to be elevated in salivary gland tissue and in peripheral blood in pSS (180)

IL-17 is a proinflammatory cytokine mainly produced by TH17 cells found both in blood and salivary gland tissues in pSS (180).

IL-18 works synergistically with IL-12 in stimulating T-cells, NK cells and IFN-γ production. Increased levels have been shown in blood and salivary gland tissue in pSS (181).

Treatment

To date, there are only two positive randomized studies in the treatment of pSS. Both studies entail cholinergic drugs stimulating the residual capacity of salivary and lacrimal glands (182, 183) thus ameliorating symptoms rather than affecting the inflammation. In routine clinical care, many drugs are used off label to treat various symptoms and extraglandular manifestations in pSS patients based on clinical experience from other rheumatological diseases such as SLE. These include glucocorticoids, cyclophosphamide, azathioprine, methotrexate, mycophenolate mofetil, hydroxychloroquine and rituximab.

Hydroxycholoroquine (HCQ)

Hydroxycholoroquine is a widely used drug for the treatment of e.g. arthritis and cutaneous manifestations of pSS. HCQ treatment is shown to be effective in the treatment of SLE and RA (184, 185). HCQ binds to DNA in endosomes and inhibits the binding of nucleic acids to TLR7 and thus the induction of IFNs (186). In pSS, minor open-label studies have shown effects on dry eye symptoms, salivary flow, pain, and BAFF levels (187-189). There are also reports of a lower frequency of EGM amongst pSS patients treated with HCQ (190). Despite this, larger double-blind clinical trials have failed to show any effect (191-193).

TNF-α-blockers

TNF-α-blockers have been investigated in pSS with negative results (194, 195).

Rituximab

Rituximab is a monoclonal chimeric antibody against the CD20 epitope on B-cell causing depletion of B-cells from early pre-B-cells to more mature B-cells except plasma cells. Originally designed as a treatment for lymphoma, it has later proved effective in rheumatological conditions such as rheumatoid arthritis and small vessel vasculitis (196, 197). It is also used off label in SLE, although clinical trials have failed, probably partly due to study design (198). In pSS, there are many reports of beneficial effects (199-201) although two larger double blind clinical trials failed to

meet their primary endpoint (202, 203).By analogy to the trials in SLE, this may be due to study design and choice of the primary endpoint.

Belimumab

The anti-BAFF antibody belimumab has been studied in a phase II clinical study in pSS with systemic complications, with promising results (169). A follow-up study, evaluating a minority of patients from the study 12 months after the end of the study found that patients were deteriorated with increased ESSDAI scores implying that the treatment halted the progression of the disease (204).

Rituximab combined with Belimumab

After treatment with rituximab, an increase of BAFF has been noted (205). Due to the potential negative effect by BAFF on stimulation of B-cells and the effect of anti-BAFF in previous studies, a clinical study has been initiated investigating combination treatment with rituximab and belimumab (clinicaltrials.com: NCT02631538).

Ianamumab

The BAFF-receptor antagonist Ianamumab, causing B-cell lysis has been studied in a small study with limited beneficial effects (206) and a phase II study is ongoing (clinicaltrials.com: NCT02962895).

Abatacept

To be activated, the T-cell has to bind to an APC with both the T-cell receptor (to MHC I) and the co-receptor CD28 (to CD80/86). Another transmembrane molecule on the T-cell, CTLA-4, can also bind to CD 80/86 and acts as a natural inhibitor of activation. Abatacept is a fusion protein that consist of the Fc part of the IgG1 and the extracellular domain of CTLA4 and thus inhibits T-cell activation. Two small open label studies in pSS has been performed showing reduced germinal centre formation and increased salivary flow (207) as well as a reduced ESSDAI and ESSPRI scores in abatacept-treated patients (208). Two ongoing phase III studies have not yet reported any results (clinicaltrials.com: NCT02067910 and NCT02915159).

Filgotinib/tirabrutinib/GS-9876

A clinical trial investigating the effect of filgotinib (JAK1 inhibitor), tirabrutinib (BTK inhibitor) and lanraplenib (SYK inhibitor) in pSS are currently ongoing (clinicaltrials.gov: NCT03100942).

Tofaticinib

The JAK1/3-inhibitor tofacitinib, administered in eye drops has been investigated in patients with dry eye disease (thus not formally pSS patients) with positive results and better tolerability than cyclosporine eye drops (which are occasionally used for severe dry eye disease in pSS) (209).

Iscalimab

A phase II study with the anti CD40L Iscalimab has recently been completed (clinicaltrials.gov: NCT 02291029) with positive results and a phase III study is planned (clinicaltrials.gov: NCT 03905525).

In summary

In clinical care, many drugs are used off label to treat extraglandular manifestations. Although larger clinical trials of various immunomodulatory drugs have failed to reach their endpoints so far, which can partly be due to patient selection and/or the instruments chosen to measure effect, some results are promising. The accelerated development of new drugs targeting specific immunological pathways in combination with an increased understanding of the pathogenesis of pSS and other autoimmune diseases is encouraging. Hopefully, more effective drug regimens will be developed in the coming decade.

Aims

The aims of the studies presented in this thesis were:

- To explore potential epidemiological risk factors for pSS by analysing prediagnostic epidemiological data from two large population-based health surveys: the Malmö Preventive Medicine Programme and Malmö Diet and Cancer studies.
- To evaluate the interference caused by rheumatoid factor/heterophilic antibodies in a multiplex bead-ELISA platform.
- To explore the effect of smoking on cytokine patterns and phenotype in pSS patients.
- To explore the association between smoking and pSS phenotype (as measured by ESSDAI and ESSPRI) and type I interferon signature.

Material and methods

Subjects

Malmö Sjögren's syndrome Registry (MSSR)

The MSSR was founded in 1984 by associate professor Rolf Manthorpe as a basis for studies in pSS. Clinical data important for pSS including the presence of anti-SSA/SSB, RF, complement factors, results of sialometries, Schimer's tests and lower lip biopsies as well as date of diagnosis have been registered ever since. Initially, the Copenhagen criteria (24) was used for classifying patients. Since 1993, the European criteria (27) was used in parallel and since 2002 also the AECG criteria (28). Currently, the patients are classified according to the AECG criteria and the ACR/EULAR criteria (210) in parallel. At the time of writing this thesis, the registry entails 489 patients, of which 361 are still alive, covering over 5,500 patient years. In 2017, the registry was updated and a biobank was approved.

Patients and controls

The patients included in the studies are followed regularly at the Department of Rheumatology, Skåne University Hospital, Malmö and are included in the Malmö Sjögren's syndrome Registry (MSSR).

Study I included 63 pSS patients from the MSSR (median age 53 years (q1–q3: 49-56); 58 females) that prior to pSS diagnosis had participated in either the Malmö Preventive Medicine programme (MPMP, 34 patients) or the Malmö Diet and Cancer Study (MDCS, 29 patients). 252 controls, matched for sex, age and time of inclusion were obtained from the corresponding health surveys. At the time of the study, the MSSR included 409 pSS patients.

Study II included 14 patients with rheumatoid arthritis (RA) (age 64 years (SD +/-9); 11 females) previously included in an open label trial with adalimumab (ClinicalTrials.gov, NCT01270087). The patients were all consecutive patients seen at the Department of Rheumatology Malmö, fulfilling the 1987 American College of Rheumatology (ACR) criteria for RA, (211) and for whom treatment with adalimumab was indicated according to their rheumatologist. They had to be non-responders to at least one disease-modifying anti-rheumatic drug (DMARD)

and have an active disease (at least 6 swollen joints in the 28-joint index, and a CRP >8 mg/L within the past 3 months). Patients were excluded if they had been treated with anti-TNF drugs in the last three months prior to inclusion, received intravenous corticosteroids within fourteen days before inclusion, and if they had ongoing treatment with high-dose oral corticosteroids (equivalent to ≥20 mg of prednisolone daily) or had completed such treatment less than fifteen days before inclusion. Additionally, sera from four patients with RA and known high levels of RF and from two RF positive patients with pSS were included in the protocol developing part.

Study III included 51 consecutive pSS patients (median age 61years (q1–q3: 52–69); 49 females). Patients were recruited amongst patients followed regularly at the Dept of Rheumatology in Malmö and all participated in the MSSR. Clinical and laboratory parameters were assessed, the ESSDAI scores were calculated and patients filled out the ESSPRI form. Thirty-three population controls from Malmö or its surroundings (median age 47 years (q1–q3: 39–61); 19 females) were randomly selected from the City of Malmö or its surroundings using the Swedish population registry.

Study IV included 90 consecutive pSS patients (median age 63 (q1–q3: 52–73); 84 females) regularly followed at the Dept of Rheumatology in Malmö. All participating in the MSSR.

44 of the 90 patients included in Study IV were also included in Study III.

Methods

Patient identification and selection of variables in Study I

In **Study I** the MSSR patient cohort was linked to two large population-based prospective health surveys, the MPMP and the MDCS:

Malmö Preventive Medicine Programme (MPMP)

The MPMP was a case finding program for cardiovascular disease and alcohol abuse performed in Malmö between 1974 and 1991. The aim of the study was to screen a substantial part of the adult population and identify high-risk individuals for preventive intervention. The study entails 33,346 individuals. Of these, 22,444 were men born between 1921 and 1938 and 10,902 were women born between 1925 and 1938. Individuals born and still living in Malmö were invited to participate in the study. The overall participation rate was 71%. The population of Malmö was 246,000 in 1974 (men 119,00, women 128,000). The current population (2018) is

339,000. In the study, blood samples were stored and biometric data was registered (including height, weight and blood pressure). Additionally, patients filled out an extensive form including information on diseases in their family, occupation, diet, reproductive factors, education and medication (212, 213).

Malmö Diet and Cancer Study (MDCS)

The aim of MDCS was to study the effect of diet on cancer development and mortality (214). The primary source population was, in 1991, defined as all inhabitants living in Malmö born during 1926–1945. The cohort was extended in May 1995 to include all women born during 1923–1950 and all men 1923–1945. This extension gave the cohort a total source of population of 74,138 persons. The total population of Malmö at that time (1991) was 235,000. Base-line examinations took place between March 1991 and October 1996. The only exclusion criteria were inadequate Swedish language skills and mental incapacity. Detailed information on the recruitment is described elsewhere (215). Information on lifestyle factors including smoking, level of education, reproductive factors and previous and current health status were obtained using a self-administered questionnaire. A total of 30,447 individuals were examined and thus the participation rate was 41%.

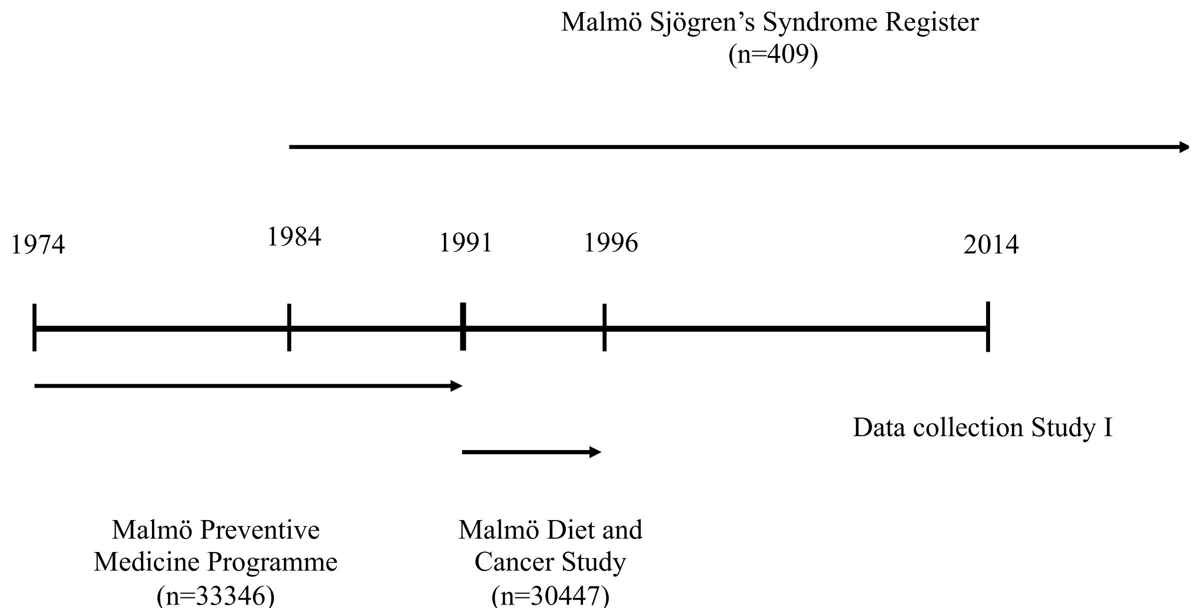

Figure 3.
Inclusion of participants in Study I

Selection of variables

Due to the low numbers of pre-pSS patients in each study, the lists of variables in MPMP and MDCS were compared to identify identical variables or variables that could be translated into a common variable. The following variables were identified:

a) Smoking status

   Patients and controls were classified as current/not current smokers at the time of participation in the health surveys, and also as current/former/never smokers.

b) Level of formal education

   In the MDCS, formal education was classified as follows: ≤8 years of elementary school, 9–10 years, 11–12 years, >12 years and university degree. In the MPMP, information was available on whether the individual had attended elementary school, secondary school or higher education. A low level of formal education was classified in the MDCS as ≤8 years and in the MPMP as elementary school only.

c) Socioeconomic status

   Information on socioeconomic status had previously been incorporated in MPMP and MDCS by linking the databases with data on standardized socioeconomic status by occupation, based on information from Statistics Sweden, as previously described in detail (216). The categorization of socioeconomic status by Statistics Sweden includes a division into groups corresponding to working class, middle class, and a group of self-employed inhabitants. For the present study, participants were classified as blue-collar workers or white-collar workers. Blue-collar workers included both skilled and unskilled manual workers, corresponding to the working-class group from Statistics Sweden. White-collar workers included non-manual employees of high level (occupations normally requiring six years of post-comprehensive school education, e.g. higher civil servants and executives), medium level (occupations normally requiring three years but not six years of post-comprehensive school education, with or without subordinates) and low-level (normally requiring two but not three years of post-comprehensive school education with or without subordinates) and self-employed professionals such as architects and lawyers.

d) pSS specific variables

   pSS specific variables such as time of diagnosis, time of symptom onset, autoantibody positivity, and lip biopsy positivity were extracted from the MSSR.

Patient evaluation and cytokine analysis in Study II

Patient evaluation

RA patients were evaluated at baseline and after 3 months using standard measures: number of swollen and tender joints (using the 28-joint index), C-reactive protein, RF, erythrocyte sedimentation rate (ESR), Health Assessment Questionnaire Disability Index (HAQ-DI), patient's assessment of pain, and patient's global assessment of disease activity.

Cytokine analyses

Cytokine analyses were performed using a custom made commercial 12-plex kit (IL-1b, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-15, IL-17, IFNγ, TNFα) from BioRad using a Luminex 200 xMAP system (Luminex, Austin, TX, USA).

To block interaction with heterophilic antibodies, HeteroBlock (Omega Biologicals, Bozeman, MT, USA), a mix of purified IgG from animal sources that has been shown to block activity of heterophilic antibodies in previous studies (217-219), was used. To determine the optimal concentration of HeteroBlock, four patients with RA and known high RF levels, two RF-positive patients with Sjögren's syndrome, and one healthy control were analysed with different concentrations of HeteroBlock (0, 160, 1600, 3,200 µg/mL serum) guided by previous results in the literature (217). HeteroBlock was added to the serum and samples were incubated for 30 minutes with shaking at 300 rpm. To evaluate the potential interference of HeteroBlock in the assay, the control serum was analysed with the same concentrations of HeteroBlock (0, 160, 1600, 3200 µg/mL serum). Additionally, a cytokine mix (Bio-Rad, Hercules, CA, USA) was added to the control serum at two different concentrations and then analysed with three different concentrations of HeteroBlock (160, 1600, 3200 µg/mL serum). After blocking and spiking with cytokines, samples were analysed using a custom- made bead ELISA 12-plex kit [IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-15, IL-17, interferon (IFN)-γ, TNF-α] from Bio-Rad on a Luminex 200 xMAP system (Luminex, Austin, TX, USA). The analysis was performed according to the protocol provided by the manufacturer. A standard curve with two additional dilution steps compared to standard was chosen to catch low values. All samples were analysed in duplicate, and mean values were used. Data analysis was performed using Bio-Plex Manager software version 6.1 (Bio-Rad).

Blood samples from RA patients in the adalimumab study were obtained in a standardized fashion: After centrifugation (3000 rpm, 10 min), serum was removed and stored at –80°C until analysis. The mean storage time before analysis was 7.5 years. Baseline samples were thawed twice before the present analysis for pilot studies of the assay. Follow-up samples had not been thawed previously. Before

analysis, samples were thawed and centrifuged at 14,300 rpm for 20 seconds to reduce debris.

Serum samples from the 14 patients included in the study were analysed using the 12-plex kit mentioned above. Samples were analysed both with and without Heteroblock (1600 µg/mL serum) based on the preceding analysis of optimal concentration. All samples were run in duplicate and mean values were used. The assay was performed according to the protocol provided by the manufacturer. Data analysis was performed using Bio-Plex Manager software version 6.1 (Bio-Rad).

Patient evaluation and cytokine analyses in Study III

Patient and control evaluation

At inclusion, routine laboratory analyses and physical examination were performed after which ESSDAI values were calculated and the patients filled out the ESSPRI form and a questionnaire on smoking habits including mean cigarette consumption. When investigating the effect of smoking on the presence of positive lip biopsy and of SSA/SSB antibodies, smoking status at the time of diagnosis was used, since the biopsies and analysis of SSA/SSB antibodies were performed at the time of diagnosis. The patients were also investigated for presence of chronic obstructive pulmonary disease (COPD) and interstitial lung disease (ILD) as part of another study, as previously reported (220). Briefly, COPD was defined according to the Global Initiative for Lung Disease (GOLD) criteria (221) and ILD was defined as the presence of ground glass attenuation, traction bronchiectasis, or honeycombing in high-resolution CT scans.

Population-based controls, living in the City of Malmö or its surroundings were randomly selected from the Swedish population register and asked via mail whether they were willing to participate in the study. If informed consent was received, the subject was invited to the Department of Rheumatology outpatient clinic, where data on age, sex, medical history, medication, present pregnancy and current smoking were registered, and a physical exam was performed.

Cytokine analyses

Serum samples from patients and controls were obtained and stored at -80ºC until analysis. The serum samples were analysed with four different panels, all using the Meso-Scale platform. Panel 4 was purchased from Meso-Scale for Interferon-α2a (IFN-α2a Ultra-sensitive kit, K151ACC). Panel 1–3 were assays made in-house. Panel 1 included B-cell activating factor (BAFF), Epidermal Growth Factor (EGF), Fas-ligand, Interleukin-3 (IL-3), IL-33, Regulated upon Activation, Normal T-cell Expressed and presumably Secreted (RANTES), and Transforming growth factor b 1 (TGF-b1). Panel 2 included IFN-γ, IL-2, -6, -8, -10, -12, -17, -18, -1b, Tumour Necrosis Factor-α (TNF-α). Panel 3 was IL-4. For the in-house assays, the

plates were coated using U-plex development kits (Meso-Scale 10-plex K15235N for panel 2, 7-plex K15232N for panel 1, 2-plex K15227N for panel 3). The different capture antibodies were first biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (ThermoFisher, 21327), then bound to different linkers 1-10, mixed together to the concentrations 10µg/ml per antibody, added to each plate well (50µl/well) for all plates in the respective panels, and incubated for 1 hour. After washing with washing buffer (PBS containing 0.05% Tween 20), the plates were stored at 4°C until use. Detection antibodies were sulfo-tagged using MSD Gold Sulfo-tag NHS-Ester (Meso-Scale, R91AO-2). All antibodies were purchased from RnD systems. For panel 1, 2 and 3, samples were diluted 1:1 in diluent 7 (containing proteins, blockers and preservatives, Meso-Scale, R54BB) and pipetted in duplicates (25µlx2) on the plates together with calibration curves and high and low controls. Calibrators and controls were prepared by recombinant antigens diluted in diluent 7. The plates were sealed and incubated with shaking for 2 hours, washed 3 times, the corresponding detection antibodies (25µl to each well, conc. 0,1µg/ml) were added, and incubated with shaking for 2 hours. Finally, the plates were washed, 150µl 2xRead buffer T (Meso-Scale R92TC) was added per well, and immediately read at the QuickPlex reader. For panel 4, analysis was made as instructed in the kit insert. Concentrations were calculated with Discovery Workbench software (Meso-Scale) from calibration curves using 4 parameter logistic fit. Based on previous reports (219, 222), the panels were assessed for proneness to interaction with heterophilic antibodies/rheumatoid factor (RF) using pooled IgM/IgA RF positive sera and pooled healthy control sera with and without HBR Plus (Scantibodies Laboratory, Santee, CA, USA) without any significant difference in cytokine levels (Table 5). Subsequent analyses were performed without any additional blocker than what was added in diluent 7 by the manufacturer.

Table 5.
Difference in cytokine detection signal in pooled healthy control sera and pooled RF positive sera with and without HBR plus

	Control pool	**Control pool + HBR plus**		**RF sera pool**	**RF sera pool + HBR Plus**	
	Signal	Signal	Ratio	Signal	Signal	Ratio
BAFF	737	810	1.10	737	817	1.18
EGF	26061	24602	0.94	32823	32052	0.98
FAS Ligand	2843	2558	0.90	3056	2690	0.88
IL-3	788	541	0.69	586	646	1.10
Il-33	425	300	0.71	367	303	0.83
RANTES	779085	754518	0.97	774985	741468	0.96
TGF-β1	4085	5153	1.26	2845	3604	1.27
IFN-γ	95	71	0.75	81	83	1.02
IL-10	155	157	1.01	177	171	0.97
IL-12	92	89	0.97	96	105	1.09
IL-17	94	80	0.85	117	92	0.79
IL-18	2294	4082	1.78	4438	6809	1.53
IL-1β	94	103	1.10	108	107	0.99
IL-2	70	79	1.13	99	100	1.01
IL-6	116	157	1.35	149	198	1.33
IL-8	796	714	0.90	861	752	0.87
TNF-α	134	143	1.07	248	181	0.73
IL-4	88,8	87,3	0.98	87,5	80,2	0.92

Patient evaluation and type I interferon signature analysis in Study IV

Patient evaluation

At inclusion, routine laboratory analyses and physical examinations were performed in pSS patients, after which ESSDAI values were calculated. Additionally, patients filled out the ESSPRI form and a questionnaire on smoking habits. The questionnaire on smoking habits included questions on whether the patient had ever been a regular smoker and, if the patient declared they had been a regular smoker, if they had given up smoking. The patient was considered a current smoker if they answered “Yes” to the question “Have you ever smoked on a regular basis?” and “No” to the question “Have you given up smoking?”. The patient was considered a former smoker if answering “Yes” to the question “Have you ever smoked on a regular basis?” and “Yes” to the question “Have you given up smoking?”. Never smokers were defined as answering “No” to the question “Have you ever smoked

on a regular basis?" In case a patient declared ever having smoked on a regular basis, the patient was asked what year they started and stopped smoking as well a question on current tobacco consumption and mean tobacco consumption during their years of smoking (Figure 4).

Supplementary material

Smoking habits

A. Have you ever smoked on a regular basis ☐ **YES** ☐ **NO**

If YES ☐ cigarettes? If YES, when did you start? Age: ....years

☐ pipe?

☐ cigars?

B. Have you given up smoking? ☐ **YES** ☐ **NO**

If you have given up smoking, when did you give up?

Age: ....**years**

C. Your current smoking habits .... cigarettes/day

.... gr pipe tobacco/week (1 package = 50 g)

.... cigars/week

D. During the whole period you have been smoking: how much have you been smoking on average?

.... cigarettes/day

.... gr pipe tobacco/week (1 package = 50 g)

.... cigars/week

Figure 4.
Questionnaire on smoking habits (translated)

Patients were divided into the following groups according to smoking status: i) current smokers ii) former smokers and iii) never smokers. Cigarette consumption, defined as pack-years was also calculated from the questionnaire. A pack-year is defined as twenty cigarettes smoked every day for one year, i.e. a cumulative

consumption of 7,300 cigarettes. The average number of pack-years amongst ever smokers (current+former smokers) was calculated, and patients were divided into those having a cigarette consumption above or equal to the median and those with a consumption below the median.

Laboratory analyses

Blood samples were collected during routine follow-up appointments during office hours using a standardised (non-fasting) procedure. Immunoglobulin (Ig)G, C3 and C4 were measured by nephelometry using an Immage800 (Beckham Coulter Inc., Brea, CA, USA). Data on the presence of rheumatoid factor (RF), antinuclear antibodies (ANA), anti-SSA and anti-SSB antibodies, all measured by validated methods in clinical care, were registered continuously in all pSS patients in the MSSR and were not re-analysed for the current study. Currently, RF is measured by Phadia ImmunoCap250, anti-SSA/SSB by EuroblotOne, and ANA is by indirect immunoflourescence using HEp 2010 cells as substrate.

For the IFN signature analysis, blood was collected in clotting tubes for serum preparation and in PAXgene RNA tubes (PreAnalytix, Hombrechtikon, Switzerland) for whole blood RNA analysis. RNA isolation, cDNA preparation and RT-PCR were performed according to the manufacturer's protocol. The protocol and selection of expressed genes was previously developed for another study (48). The type I IFN induced genes analysed include IFI44, IFI44L, IFIT1, IFIT3 and MxA. The expression of the genes was summarised in order to create an IFN-score. Patients were divided into groups that were positive or negative for type I IFN using a threshold of mean healthy controls (HC) + 2 $S.D_{HC}$ based on the previous analysis (48).

EULAR Sjögren's syndrome disease activity index (ESSDAI) and EULAR Sjögren's syndrome Patient Reported Index (ESSPRI)

The ESSDAI and ESSPRI indices were used in **Study III and IV** to assess the disease activity and burden of symptoms amongst the patients. Both indices are presented in detail in the introduction of this thesis.

Statistics

In **Study I** potential predictors of pSS were analysed using conditional logistic regression. Each case and the corresponding four controls were given a group number which was entered into the logistic regression as a categorical variable. The two-sided Fisher's test was used to analyse differences in immunological markers after diagnosis of pSS by smoking status at the inclusion in the MPMP or MDCS.

A p-value <0.05 was considered significant. The statistical analyses were performed using SPSS version 22 for Mac.

In **Study II**, the difference in clinical parameters and cytokine levels between baseline and 3 months were analysed with the paired-T-test for normally distributed variables and the Wilcoxon signed rank test for non-normally distributed variables. Spearman's rank test was used to assess correlations between absolute values as well as between changes over time in cytokine levels and clinical parameters. The statistical analyses were performed using SPSS version 20 for Mac.

In **Study III** the Mann-Whitney U-test was used for analysing differences in cytokine level between patients and controls. The Spearman's rank correlation test was used for correlations. The Chi^2-test was used for the comparison of discrete parameters. The statistical analyses were performed using SPSS version 22 for Mac.

Study IV: Due to the small sample sizes, continuous data was generally considered non-normally distributed and thus data are presented as median (q1–q3), the Kruskal-Wallis test was used for multiple group comparisons and the Mann Whitney U-test for comparison between groups. For continuous data including more than 80 cases, data were analysed for normality by visual inspection of histograms and Q-Q-plots as well as by the Shapiro-Wilk test. If normally distributed, data were presented as mean (+/- standard deviation [SD]), and the one-way analysis of variation (ANOVA) was used for multiple group comparisons and the independent samples T-test for comparison between groups. The Chi^2-test or Fisher's exact test were used for discrete variables. A p-value <0.05 was considered significant. The statistical analyses were performed using SPSS version 22 for Mac.

Ethics

Studies I–IV were all approved by the Regional Ethical Review Board for Southern Sweden.

Table 6.
Characteristics of patients and controls in Study I-IV

Study	Participants	Aim of study	Number (n)	Females (n)	Median age (IQR)	Selection
I	pSS patients	Study epidemiological risk factors for the development of pSS	63	48	52,6 (49; 56)	Individuals later diagnosed with pSS
	Controls		252			Controls matched for age, sex, time of inclusion
II	i) RA patients and pSS patients	i) Develop a protocol to block heterophilic antibodies/RF in bead-ELISA assay.	i) 4 RA patients and 2 pSS patients			i) RA patients with high RF-levels and RF positive pSS patients.
	ii) RA patients	ii) Analyse cytokine patterns in active RA patients before and after treatment with adalimumab	ii) 14 RA patients	ii) 11	ii) 67.3 (SD 8.9)	ii) Active RA patients
III	pSS patients	Effect of smoking on cytokine profile	51	49	61 (IQR 52; 69)	Consecutive pSS patients
	Population controls		33	19	47 (IQR 39; 61)	Randomly selected population controls
IV	pSS patients	Effect of smoking on type I IFN signature and ESSDAI/ESSPRI	90	84	62.5 (IQR 52: 73)	Consecutive pSS patients

Results and discussion

Study I

Results

Incident cases and controls

A total of 63 incident cases, patients who prior to a first diagnosis of pSS and fulfilment of the AECG criteria had been included either in the MPMP, the MDCS, or both, were identified. These are, from hereon, designated as “pre-pSS patients”. 252 matched controls were obtained from the corresponding health surveys. Characteristics of the patients at diagnosis are shown in Table 7. The median time between inclusion in one of the health surveys and subsequent pSS diagnosis was 8.2 years (q1–q3: 2.4–14.1). The median age at inclusion in the health surveys was 51 years, and the median age at pSS diagnosis was 61 years. Demographics and information on exposures for cases and controls are listed in Table 8.

Table 7.
Characteristics of incident cases of primary Sjögren’s syndrome

Characteristic	Patients
Sex (males/females) (n)	5/58
Age at diagnosis of pSS (years)	61 (54–69)
Time between inclusion and diagnosis (years)	8.2 (2.4–14.1)
TIme between onset of symptoms and diagnosis (years)	4.0 (1.0–6.8)
Anti-SSA seropositives	59%
Anti-SSB seropositives	41%
ANA seropositives	73%
RF seropositives	57%
Lower-lip salivary gland biopsy focus score ≥ 1	85%

Results are presented as numbers, median (IQR) or % in cases with available data. Missing data on time of symptom onset in 15 patients, anti-SSA, anti-SSB and ANA in 1 patient, RF in 2 patients and lower lip salivary gland biopsy focus score in 6 patients

Smoking and the risk of pSS

Information on smoking status was available in 60 cases. The distribution of current, former and never smokers differed between controls and pre-pSS patients with a higher proportion of former smokers amongst pre-pSS patients (Table 8). In conditional logistic regression analysis, current smoking at the time of inclusion in the health surveys was associated with a significantly lower risk of later being diagnosed with pSS compared to current non-smoking (OR 0.3; 95% CI 0.1–0.6). In addition, former smoking was associated with a higher risk of later being diagnosed with pSS, both compared to never smoking (OR 4.0; 95% CI 1.8–8.8) and current smoking (OR 7.8; 95% CI 3.1–19.9) (Table 9).

Table 8.
Demographic and exposure information in pre-primary Sjögren's syndrome cases and controls

Characteristic	Cases (N=63)	Controls (N=252)	*P*-value**
Female sex; n (%)	58/63 (92)	232/252 (92)	1.00
Age at inclusion in the population survey; mean (IQR), years.	52.6 (49.0-56.0)	52.7 (49.0-56.0)	0.94
Current smoker; n (% *)	10/60 (17)	92/251 (37)	0.003
Former smoker; n (%*)	32/60 (53)	63/251 (25)	<0.001
Never smoker; n (%*)	18/60 (30)	96/251 (38)	0.23
White-collar worker; n (%*)	26/54 (48)	131/236 (56)	0.33
Blue-collar worker; n (%*)	22/54 (41)	89/236 (38)	0.68
Other socioeconomic status; n (%*)	6/54 (11)	16/236 (7)	0.28
Low level of formal education; n (%*)	25/58 (43)	99/243 (41)	0.73
Medium/high level of formal education n (%*)	33/58 (57)	144/243 (59)	0.73

*Percentage of individuals with available data. **Mann-Whitney U-test

Sensitivity analyses

Retrospective data from the MSSR on symptom onset was available in 48 cases. There were 28 patients who had symptom onset >1 year after inclusion in the population surveys (range 2–24, median 6.5, IQR 3.25–14.75), and information on smoking status was available for 26 of these patients. In analyses restricted to these cases and their controls, current smoking was still associated with a lower risk of later being diagnosed with pSS compared with current non-smoking [OR 0.2 (0.1–0.9)], but former smoking was not significantly associated to the diagnosis of pSS although a positive OR [OR 1.7 (0.6–5.6)] (Table 9). In order to answer the question as to whether cessation of smoking was due to symptoms of pSS, we analysed the time window between symptom onset and cessation of smoking. Amongst the cases with symptom onset >1 year after inclusion in the population surveys, there were 12 patients who were former smokers. The time period between cessation of smoking and symptom onset was >5 years in all these cases.

In the whole group of pre-pSS patients who were former smokers and for whom information was available at symptom onset (n=23), including those with symptom onset before inclusion in the health surveys, cessation of smoking occurred >5 years before symptom onset in 20 cases, 4 years before symptom onset in 1 case and after symptom onset in 1 case.

Socioeconomic status, level of formal education and the risk of pSS

Socioeconomic status, comparing blue-collar workers and white-collar workers, did not affect the risk of being diagnosed with pSS. Furthermore, there was no association between the level of formal education and subsequent diagnosis of pSS (Table 9).

Stratified analyses

To further examine the relationship between smoking or other exposures and pSS, analyses were performed separately in cases with a time period between inclusion in one of the health surveys and diagnosis of pSS that was above or below the median (8.2 years) and in the corresponding controls. The results in these subgroups were similar to those in the whole group (Table 10).

Anti-Sjögren's syndrome A (anti-SSA/Ro) antibody positivity or presence or absence of focal sialoadenitis at the time of diagnosis was not significantly different between individuals who were currently or not currently smoking at the time of inclusion in the health surveys, nor between those who had ever been smoking or never smoking, nor those who were currently smoking, were former smokers, or had never been smoking (Table 11).

Table 9.
Association between smoking status, socioeconomic status and level of formal education and development of primary Sjögren's syndrome

		All pSS cases (n=63) and controls (n=252) OR (95% CI)	Exposed (cases/controls)	pSS cases with documented symptom onset after inclusion (n=28) and matched controls (n=112) OR (95% CI)	Exposed (cases/controls)
Smoking	Not current smokers	Reference	50/159	Reference	22/74
	Current smokers	0.3 (0.1–0.6)	10/92	0.2 (0.1–0.9)	4/38
	Never smokers	Reference	18/96	Reference	10/43
	Former smokers	4.0 (1.8–8.8)	32/63	1.7 (0.6–5.6)	12/31
	Current smokers	0.5 (0.2–1.3)	10/92	0.3 (0.1–1.5)	4/38
Socioeconomic status	White-collar worker	Reference	26/131	Reference	11/53
	Blue-collar worker	1.4 (0.7–2.8)	22/89	0.8 (0.2–2.5)	9/40
	Other	2.4 (0.7–8.4)	6/16	1.0 (0.3–3.2)	4/11
Education	Medium/high	Reference	33/144	Reference	11/55
	Low	1.1 (0.6–2.1)	25/99	1.4 (0.5–4.0)	13/49

Smoking status and level of formal education were assessed at inclusion in the health surveys. Patients were subsequently diagnosed with primary Sjögren's syndrome (pSS) after a median of 8.2 years (q1–q3: 2.4–14.1) after inclusion

Table 10.
Associations stratified by time from inclusion to diagnosis of primary Sjögren's syndrome

		Time period ≤8.2 years (n = 32) OR (95% CI)	Exposed (cases/controls)	Time period >8.2 years (n = 31) OR (95% CI)	Exposed (cases/controls)
Smoking	Not current smokers	Reference	27/87	Reference	23/72
	Current smokers	0.3 (0.1–0.9)	4/40	0.3 (0.1–0.8)	6/52
	Never smokers	Reference	11/58	Reference	7/38
	Former smokers	4.9 (1.6–15.2)	16/29	3.4 (1.1–10.8)	16/34
	Current smokers	0.5 (0.1–1.8)	4/40	0.5 (0.1–2.0)	6/52
Socioeconomic status	White-collar worker	Reference	13/65	Reference	13/66
	Blue-collar worker	1.4 (0.5–3.8)	11/51	1.3 (0.5–3.7)	11/38
	Other	2.0 (0.3–14.3)	2/6	2.6 (0.5–14.4)	4/10
Education	Medium/high	Reference	18/74	Reference	15/70
	Low	1.1 (0.4–2.5)	13/53	1.1 (0.4–3.2)	12/46

Table 11.
Immunologic markers at primary Sjögen's syndrome diagnosis or later by smoking status at inclusion n (% of available)

	Current smoker	***P*-value***	**Former smoker**	***P*-value***	**Never smoker**	***P*-value***
Ant SSA positive, *n* (%)	8 (80)	0.29	18 (56)	0.60	10 (56)	0.78
Anti-SSB Positive, *n* (%)	7 (70)	0.07	11 (34)	0.43	6 (33)	0.57
ANA positive, *n* (%)	9 (90)	0.26	20 (63)	0.08	15 (83)	0.35
RF positive, *n* (%)	4 (44)	0.72	16 (50)	0.60	12 (67)	0.26
Autoantibody[a] negative	1 (10)	1.00	7 (22)	0.16	1 (6)	0.26
Focal sialoadenitis, *n* (%)	8 (80)	0.63	23 (82)	0.71	15 (94)	0.41

Smoking status at inclusion in the health surveys. Primary Sjögren's syndrome was diagnosed at a median of 8.2 years (q1–q3: 2.4–14.1) after inclusion. *Two-sided Fisher's exact test. [a]Patients who were negative for anti-Sjögren's syndrome A (SSA), anti-Sjögren's syndrome B (SSB), anti-nuclear antibodies (ANA) and rheumatoid factor (RF)

Discussion

In this study, we found no difference in the rate of ever smokers amongst the individuals later diagnosed with pSS compared to controls, but there was a significant lower frequency of current smokers and thus a significantly higher frequency of former smokers amongst pre-pSS patients. In other words, individuals later developing pSS had stopped smoking to a greater extent than individuals not developing pSS. A lower frequency of current smokers amongst pSS patients has been found in cross-sectional studies with a higher number of participants (75, 80). Due to the dryness of the mucosal surfaces in the oral cavity, airways and eyes, a potential reason for the lower frequency of current smokers is that the smoke causes more irritation, making the patients more prone to giving up. The current study gathered smoking data years before diagnosis, making it less plausible that the dryness in the oral cavity and airways is the reason for the lower frequency found in pSS populations. The sensitivity analysis, using retrospect data on dryness symptom onset from the MSSR also supports the idea that dryness was not the reason from the pre-pSS patients to give up smoking. Theoretically, the giving up smoking might have an influence on disease development. This theory was proposed by Manthorpe et al., finding a lower frequency of positive lower lip biopsies in smoking pSS patients (81). Of note, in the study by Manthorpe, the Copenhagen criteria was used and there was a similar distribution of never, former and current smokers amongst the pSS patients and controls in that study. Although smoking is associated to the development of some autoimmune diseases (86, 87), an ameliorating effect of smoking is seen in other autoimmune diseases such as Bechet's disease, Parkinson's disease, and – most striking – ulcerative colitis (87-89). The fact that smoke cessation can cause development ulcerative colitis or a worsening of symptoms is well known. The opposite is, on the other hand, true for another inflammatory bowel disease, namely Crohn's disease (87). Cigarette smoke

has both proinflammatory and anti-inflammatory effects on the immune system, which is why diverse effects on different autoimmune diseases are not surprising (91).

In contrast to studies of other chronic disorders (103, 104, 216, 223), no significant differences were found between pre-pSS patients and controls concerning socioeconomic status or education.

In conclusion, individuals later developing pSS had stopped smoking to a greater extent compared to controls. Considering that the median time between smoke cessation was long before diagnosis of pSS (8,2 years; q1–q3: 2,4–14.1) and that, in the subset of individuals with available information, the reported onset of symptoms was long after smoke cessation (4.0; q1–q3: 1.0–6.8) it is possible that either smoke cessation triggers the development of pSS or that current smoking is protective for the development of pSS.

Study II

Results

Quality of the cytokine analysis

The cytokines included in the statistical analyses of correlation to clinical outcome (IL-6, IL-7 and IL-8) were all in the declared working range of the assay according to the manufacturer (data not shown). Recovery in the analyses of baseline samples was between 82% and 129% when concentrations were higher than background apart from one cytokine, namely IFN-γ (9%). In this case, the value was below the declared working range of the assay and the fluorescence intensity value (FI-value) was very close to the blank (1.38 vs. 1.35), and thus the result was probably due to inaccuracy of the assay in the lower spectrum. In the analyses of the follow-up samples, recovery was between 69% and 139% when concentrations were higher than background (data not shown).

Data from the protocol development part

A substantial effect of HeteroBlock on measured cytokine concentrations was seen in almost all analytes except for IL-8 (Figure 5). The effect was most obvious between unblocked samples and samples blocked with HeteroBlock 160 µg/mL of serum, but in several cases, there was an additional effect when adding 1600 µg/mL. In general, no additional effect was seen when adding 3200 µg/mL of serum compared to 1600 µg/mL of serum (Figure 5). Therefore, 1600µg/mL was chosen for the subsequent investigations of sera from RA patients treated with adalimumab.

The measured cytokine levels in the control sera were stable in unspiked samples and in those spiked with different cytokine concentrations, regardless of the concentration of added HeteroBlock (Figure 6).

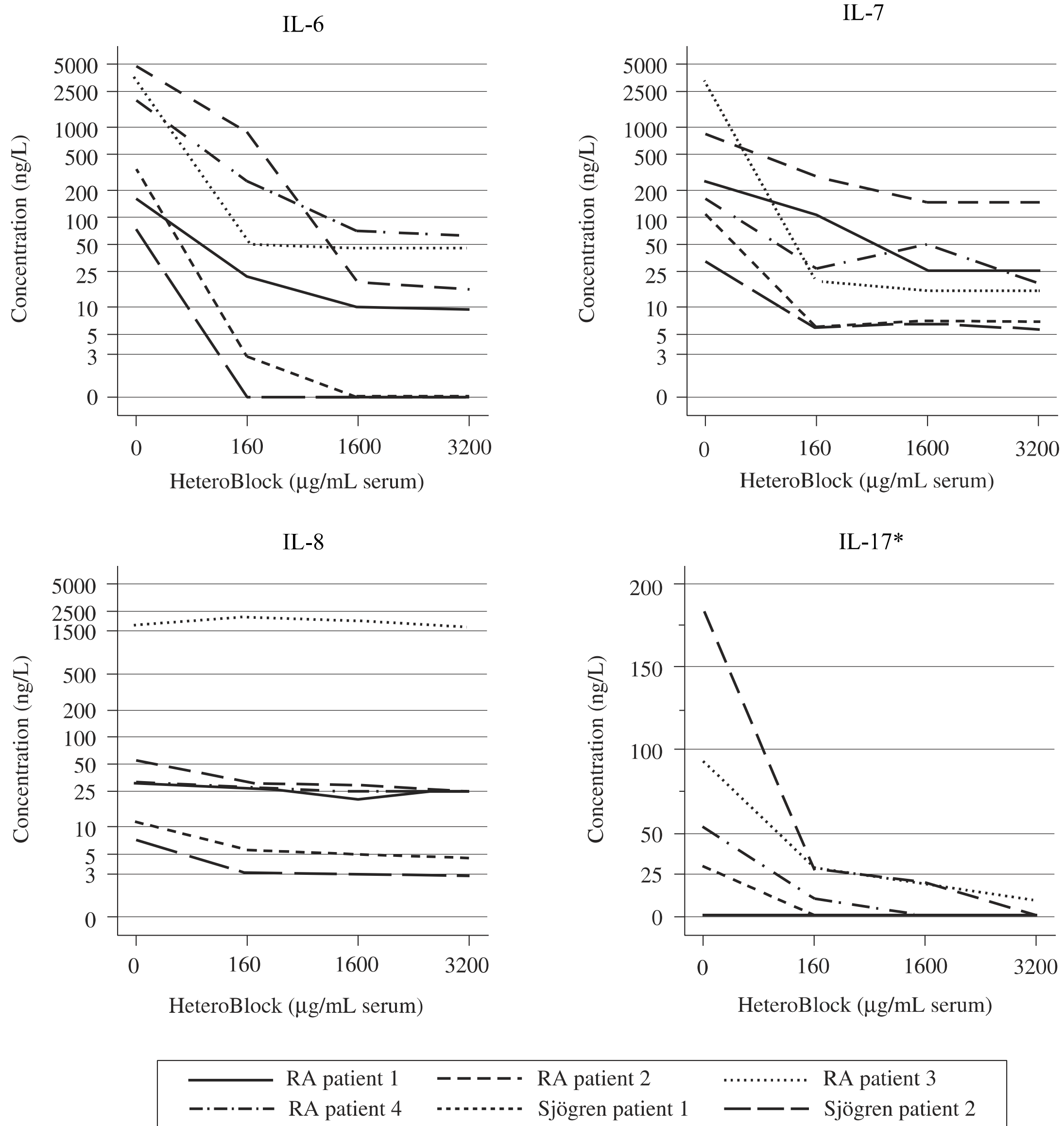

Figure 5. Measured cytokine levels in representative examples of serum from four RA patients and two pSS patients with different concentrations of HeteroBlock

Logarithmic scale unless otherwise indicated. *Linear scale due to different distribution.

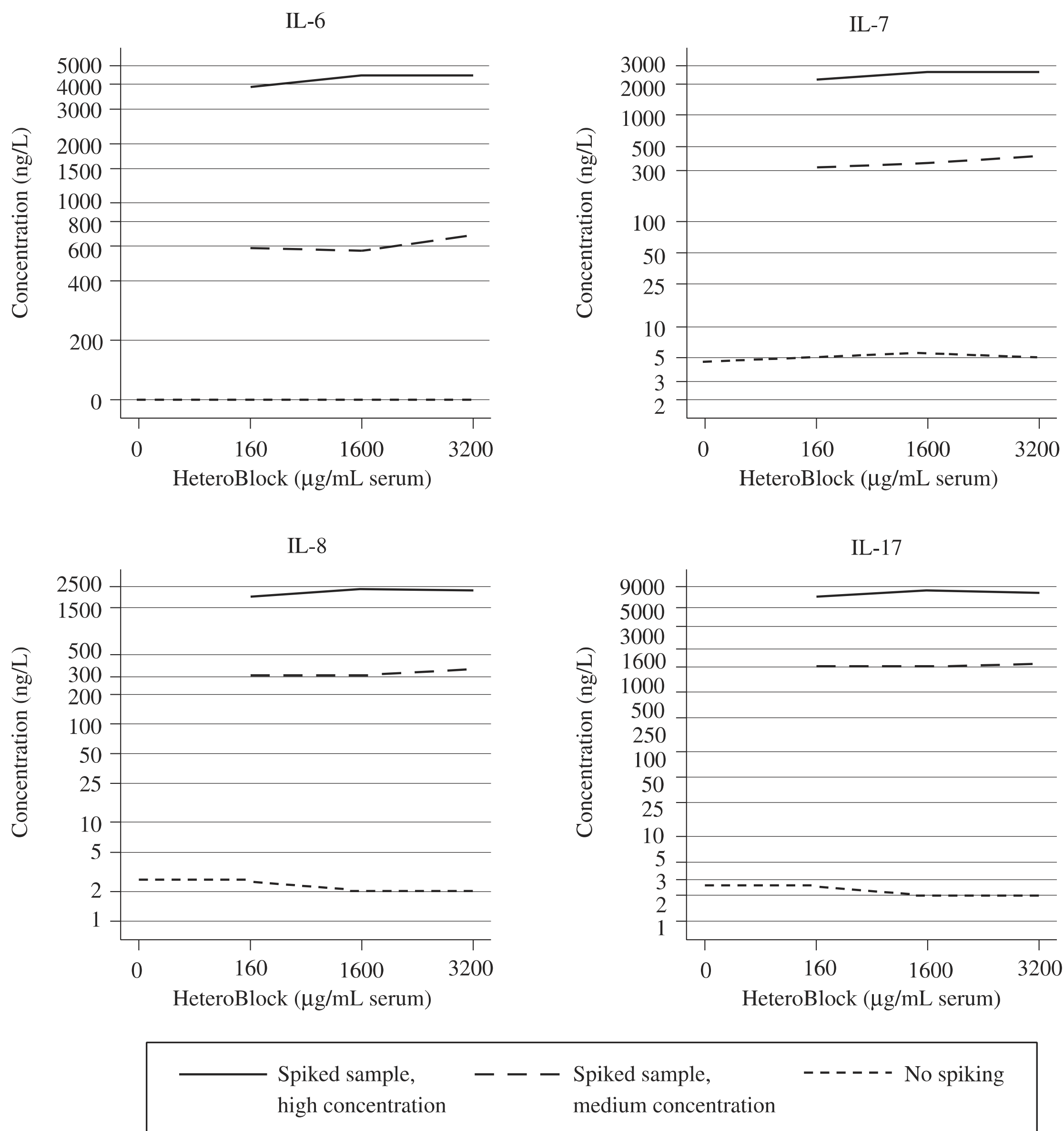

Figure 6. Control serum spiked with different concentrations of cytokines analysed with addition of different concentrations of HeteroBlock.
Data for RA patients at baseline and after 3 months with adalimumab treatment

Data for RA patients at baseline and after 3 months with adalimumab treatment

Clinical baseline characteristics: Fourteen patients with active RA (Table 12) were started on treatment with adalimumab 40 mg subcutaneously every 2 weeks. Eight patients were on MTX on a mean dose 18.75 mg/ week (range 10–25). The other six patients had previously been treated with MTX. Two of the patients had been treated with anti-TNF drugs in the past. One had stopped her only previous anti-TNF treatment just over 3 months before the start of the study. The other had received two previous anti-TNF treatments, where the last treatment was stopped more than 18 months before inclusion. Both had discontinued anti-TNF treatment

due to adverse events. Four of the patients had extra-articular involvement in the form of rheumatoid nodules at inclusion, but no current vasculitis or other severe extra-articular manifestations were recorded. One patient had a history of systemic rheumatoid vasculitis.

Table 12.
Baseline characteristics of the RA patients in the study

N	14
Gender (female/male)	11/3
Age at inclusion (years), mean (+/- SD)	63.7 (8.9)
Disease duration (years), median (q1-q39	9.0 (2.6–11.6)
RF positive n (%)	11 (78)
RF>60 IU/mL n (%)	6 (43)
RF levels (IU/mL)*, median (q1–q3)	84 (37-296)
Anti-CCP positive, n (%)	13 (93)
MTX at inclusion n (%)	8 (73)

RA: rheumatoid arthritis, RF: rheumatoid factor, anti-CCP: anti-cyclic citrullinated peptide, MTX: methotrexate, SD: standard deviation, q:quartile * In RF positive patients.

RA clinical outcomes: The disease activity score based on 28 joint counts (DAS28) decreased from baseline to the 3-month evaluation (mean 5.6 vs 4.1; $p = 0.007$). A good or moderate European League Against Rheumatism (EULAR) response was seen in 8/14 patients. Disability measured by the HAQ, and inflammation, in particular when measured by CRP, were also reduced after 3 months (Table 13). Two patients achieved clinical remission according to the EULAR criteria (DAS28 < 2.6 at the follow-up at 3 months).

Table 13.
Clinical disease severity measurements before starting adalimumab and after 3 months of treatment.

	Baseline	**Follow-up at 3 months**	**p-value**
DAS28	5.6 (1.3)	4.0 (1.4)	0.007
Swollen joint count (out of 28)	10.4 (5.4)	3.1 (3.7)	<0.001
Tender joint count (out of 28)	10.0 (8.6)	5.5 (8.3)	0.07
VAS global (mm)	55.4 (24.6)	42.4 (29.3)	0.17
VAS pain (mm)	54.0 (33.1)	35.5 (30.5)	0.10
HAQ-DI	1.48 (0.73)	1.30 (0.81)	0.22
CRP (mg/L)	22 (9-39)	8 (2-22)	0.05
ESR (mm/h)	30 (18-47)	18 (9-31)	0.10

DAS28, Disease Activity Score based on 28-joint counts; VAS, visual analogue scale; HAQ-DI, Health Assessment Questionnaire Disability Index; CRP, C-reactive protein; ESR erythrocyte sedimentation rate. Values are given as mean (standard deviation) or median (interquartile range).

Cytokine levels before and after treatment with adalimumab: When analysing samples without a blocker, the measured cytokine levels were below the working range in 115 out of 336 cytokine results. When cytokine levels were measurable, addition of HeteroBlock reduced the measured levels of cytokines in the vast majority of samples and cytokines. The maximum difference was 17 403.7 ng/L vs. non-detectable (IFN-γ). In the minority of results, where blocked values were higher than unblocked (33 out of 336 cytokine results), the difference between the results was low (maximum in absolute value 25.5 ng/L (9% of the unblocked value)); maximum in relative value 350% (14.7 ng/L)]. Samples showing the effects of blocking in one cytokine did so in all the others, with the exception of IL-8, if levels were measurable. The effect was most obvious in IFN-γ and TNF-α (Figure 7). Samples out of range, with values below the lowest point on the standard curve or below the blank, are presented as not detected.

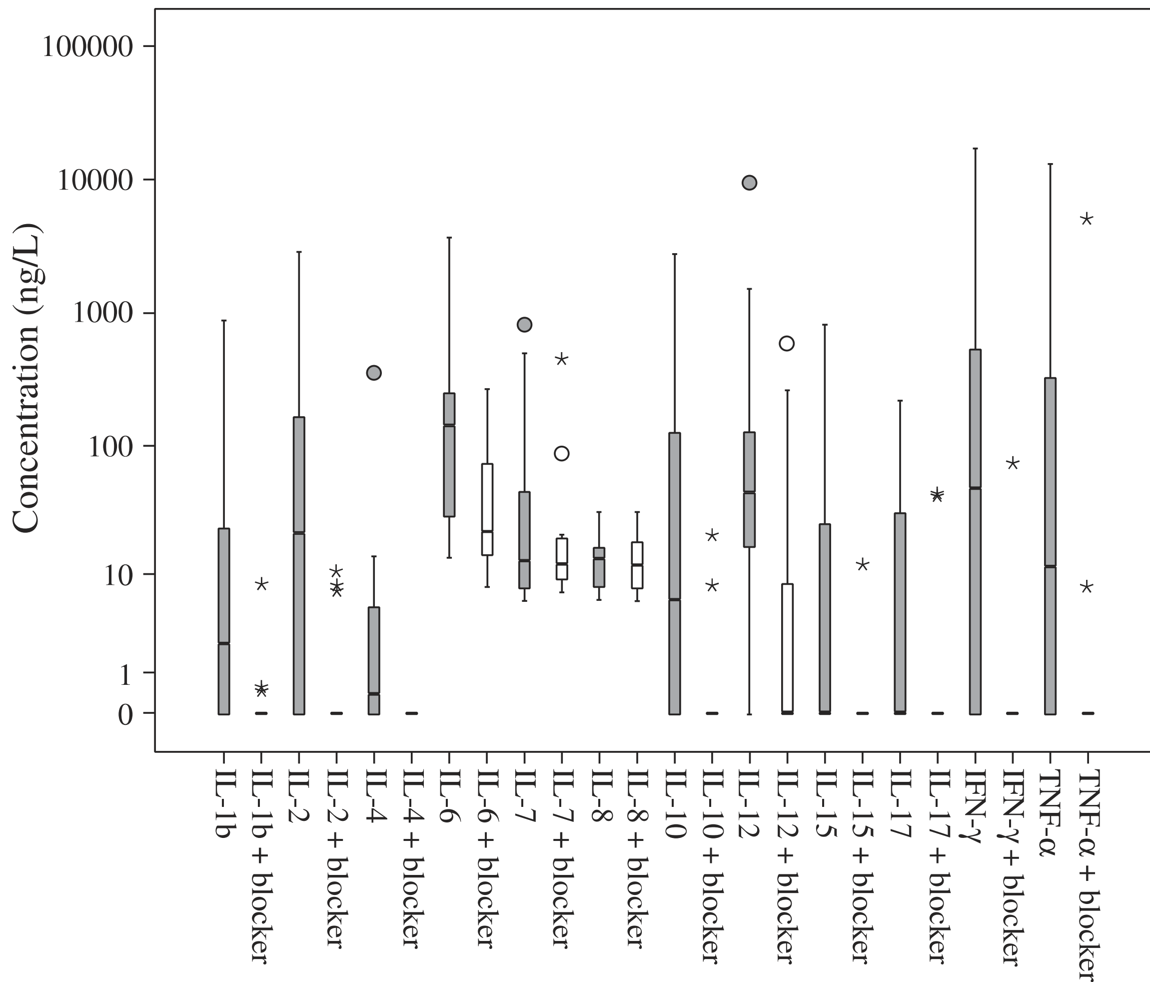

Figure 7. RA patients treated with adalimumab. Baseline samples analysed with and without HeteroBlock (1600g/µmL of serum)

Stars indicate extreme outliers, circles indicate outliers, bars indicate median and interquartile range, and whiskers indicate 95th percentile.

IL-8 seemed to be unaffected by the added blocker. The range of values for IL-8, both blocked and unblocked, was 5.8–37.2 ng/L. Blocked samples differed from unblocked samples with a median difference of 1.2 ng/L (minimum 0.1 ng/L; maximum 14.6 ng/L) between unblocked and blocked values, in baseline and follow-up samples combined. The median percentage difference [1 – (blocked value/unblocked value)] was 12% (minimum 2%; maximum 43%).

Samples with higher IgM-RF levels (> 60 IU/mL) were more affected by adding HeteroBlock (Table 14), and samples showing extreme values unblocked all had high IgM- RF levels (> 296 IU/mL). Two patients with high IgM-RF levels showed interaction comparable with those with IgM-RF levels < 60 IU/mL. Out of three IgM-RF-negative samples, two showed a clear effect of blocking and one only a marginal effect (Figure 8).

Table 14.
Change in cytokine concentration between unblocked sera and sera blocked with HeteroBlock (1600 µg/mL serum) in patients with high vs. low RF levels.

	High RF (>60 IU/mL) n=6				Low RF (<60 IU/mL) n=8		
Cytokine	Median change (ng/L)	Q1-Q3	Range		Median change (ng/L)	Q1-Q3	Range
IL-1β	14.1	0.6–253.2	-0.5–865.5		0.3	0–6.2	0–30.4
IL-2	191.3	8.5–1028.4	0–2821.0		3.3	0–78.9	0–163.9
IL-4	3.1	0–93.5	0–352.9		0	0–2.0	0–14.1
IL-6	126.7	-21.1–1211.6	-24.0–3622.9		11.1	0–95.2	-7.3–126.8
IL-7	11.7	-4.3–378.0	-14.7–405.6		1.0	-1.6–27.2	-6.2–49.3
IL-8	1.2	-0.9–4.4	-1.0–7.6		0.7	0.2–2.5	-2.5–2.7
IL-10	4.1	0–1391.7	0–2724.4		17.4	0–110.4	0–264.8
IL-12 (p70)	30.9	13.3–3157.6	0–8858.6		42.8	4.1–118.3	0–387.9
IL-15	59.4	0–302.6	0–818.8		0	0–16.5	0–25.1
IL-17	0	-3.3–76.8	-13.0–179.3		0	0–0	0–46.6
IFN-γ	333.5	14.1–5344.6	0–17403.7		12.7	0–210.8	0–1293.0
TNF-α	165.4	0–2328.9	0–7808.5		10.0	0–102.7	0–557.5

RF: rheumatoid factor

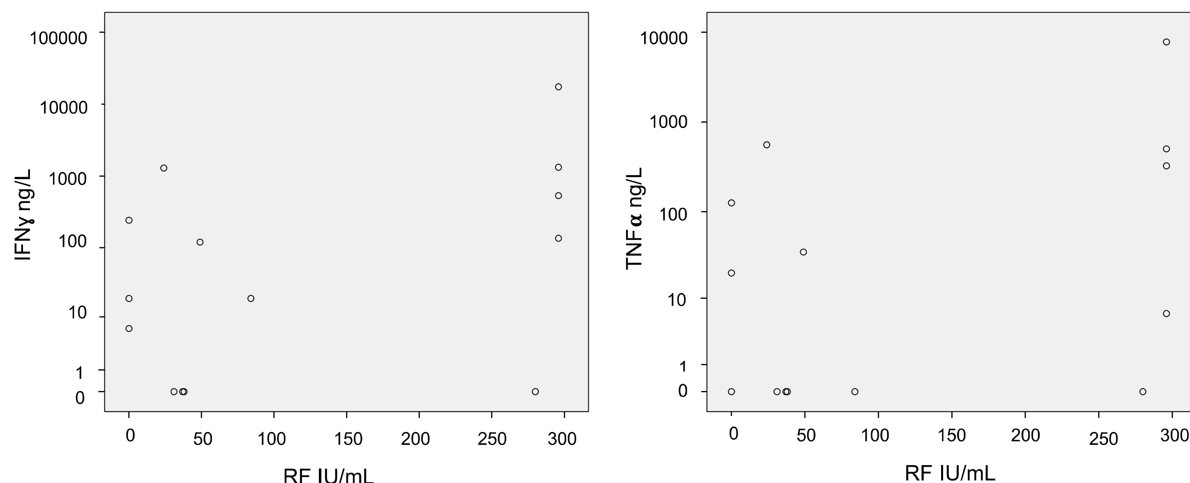

Figure 8. Difference between measured concentrations of IFNγ and TNF-α in unblocked and blocked (Heteroblock 1600 µg/ml) sera, by RF level (unblocked value – blocked value). Samples obtained from patients with RA before start of adalimumab.
RF >296 IU/ml is shown as 296, RF negative (<14 IU/ml) is shown as 0

After blocking, only IL-6, IL-7, and IL-8 had high enough measurable concentrations for the statistical analysis to be performed. Serum levels of IL-6 tended to decrease from baseline to the 3-month evaluation (p =0.24), whereas there was no major change for IL-7 and IL-8 (Table 15). When comparing baseline and follow-up samples for the individual patients, there was a difference in the ratio

between blocked and unblocked values, but the titres of IgM-RF also differed between baseline and follow-up (data not shown)

Table 15.
Measured cytokine levels (ng/L) in RA patients treated with adalimumab. All samples treated with HeteroBlock 1600 µg/mL serum.

		Baseline	3 months
IL-1b	Median (q1–q3)	nd (nd-0.1)	nd (nd-0.1)
	Min-max	nd-8.3	nd-9.1
IL-2	Median (q1–q3)	nd (nd-1.8)	nd (nd-nd)
	Min-max	nd-10.7	nd-29.5
IL-4	Median (q1–q3)	nd (nd-nd)	nd (nd-nd)
	Min-max	nd-nd	nd-nd
IL-6	Median (q1–q3)	22.0 (14.3–91.0)	5.7 (nd-67.1)
	Min-max	7.9–269.3	nd-301.0
IL-7	Median (q1–q3)	12.3 (8.9–19.9)	11.7 (9.9–15.6)
	Min-max	7.1–446.2	8.2–546.2
IL-8	Median (q1–q3)	12.0 (7.4–19.4)	10.6 (7.6–15.7)
	Min-max	5.8–31.2	6.2–33.5
IL-10	Median (q1–q3)	nd (nd-nd)	nd (nd-nd)
	Min-max	nd-20.1	nd-67.9
IL-12(p70)	Median (q1–q3)	nd (nd-14.2)	nd (nd-0.9)
	Min-max	nd-591.0	nd-1145.5
IL-15	Median (q1–q3)	nd (nd-nd)	nd (nd-nd)
	Min-max	nd-12.0	nd-52.6
IL-17	Median (q1–q3)	nd (nd-nd)	nd (nd-nd)
	Min-max	nd-43.8	nd-218.1
IFN-γ	Median (q1–q3)	nd (nd-nd)	nd (nd-nd)
	Min-max	nd-73.4	nd-nd
TNF-α:	Median (q1–q3)	nd (nd-nd)	nd (nd-nd)
	Min-max	nd-5168.7	nd-8076.0

Lower limits (ng/l): IL-1b=0.11; IL-2=0.63; IL-4=0.06; IL-6=0.11; IL-7=0.11; IL-8=0.34; IL-10=0.42; IL-12(p70)=0.55; IL-15=0.34; IL-17=1.46; IFN-γ =1.27; TNF-α: =1.47). q=quartile.

Correlation between cytokines and clinical outcome

When analysing baseline samples with no blocking agent added, there was no significant correlation between IL-6 and CRP (r = 0.19, p = 0.51). By contrast, there was a significant correlation when using blocked samples (r = 0.65, p = 0.01). The same pattern was seen when analysing follow-up samples. The decrease in IL-6, in blocked analyses, from baseline to 3 months after adalimumab initiation correlated significantly with decreases in clinically relevant laboratory markers of inflammation (CRP and ESR), whereas there was no such pattern for IL-7 or IL-8 (Table 16). In unblocked analyses, the correlation with change in IL-6 reached statistical significance for change in ESR but not for change in CRP (Table 15).

Table 16.
Correlations between changes over time in serum cytokine levels, with and without blocking, and changes in disease activity parameters*

		Change in CRP	Change in ESR	Change in DAS28
With blocking**	Change in IL-6	r=0.74 (p=0.002)	r=0.81 (p<0.001)	r=0.43 (p=0.12)
	Change in IL-7	r=0.14 (p=0.62)	r=0.39 (p=0.17)	r=-0.21 (p=0.48)
	Change in IL-8	r=0.12 (p=0.67)	r=0..37 (p=0.20)	r=0.05 (p=0.86)
Without blocking	Change in IL-6	r=0.51 (p=0.06)	r=0.69 (p=0.006)	r=0.11 (p=0.71)
	Change in IL-7	r=0.08 (p=0.78)	r=0.42 (p=0.14)	r=-0.15 (p=0.61)
	Change in IL-8	r=0.14 (p=0.65)	r=0.46 (p=0.09)	r=-0.05 (p=0.86)

*from baseline to three months after start of adalimumab; **HeteroBlock1600 µg/mL. Spearman's rank correlation.

Discussion

This study illustrates the risk of interference of heterophilic antibodies/rheumatoid factor when analysing sera from patients with RA and pSS with bead ELISA (and other sandwich ELISAs). This is a well-known problem (224), but often it is not declared as a problem, or it is not declared if the assay is tested for interference, in studies using sandwich ELISA when analysing sera from RA or pSS patients. Interaction can also cause problems in routine clinical care where ELISAs are commonly used for detection of e.g. hormones, and cancer markers (224). To avoid interference, most assays include different blockers and might also use antibodies that are less prone to interact. Since the composition of commercial assays are often proprietary, including the validation for proneness of interaction with heterophilic antibodies, the quality is often difficult to evaluate. Different strategies exist to handle heterophilic antibodies/RF, including adding mouse IgG1 (or a mix of antibodies from different species and isotypes) to the blood sample, pre-treatment with polyethylene glycol to precipitate immunoglobulins, and extraction of IgG by protein A columns (219, 224, 225). In the current study, the antibodies detecting IL-8 did not seem to be prone to interaction with RF/heterophilic antibodies and

probably one or both of the antibodies are of a different species (e.g. chicken) or of a different isotype (219).

Some limitations of this study are related to the small sample size. There was no analysis of cytokines with any other method as ‘gold standard’. Samples had been stored for a mean of 7.5 years before the analysis, which may have affected the concentration of the cytokines (226). However, the storage time was similar for baseline samples and follow-up samples. Furthermore, the baseline samples were thawed two times more than the follow-up samples. This may also affect cytokine concentrations, as shown by de Jager et al. (226). However, this effect varied for different cytokines, and in that study, IL-6 and IL-10 were stable despite several freeze–thaw cycles. This suggests that our finding of correlations between IL-6 and CRP only in the analysis including HeteroBlock would not be affected by the additional two freeze–thaw cycles of the baseline samples. The strengths of this study include the use of high concentrations of blocker to determine optimal blocking of interference, and the careful characterization of a relevant patient sample.

In conclusion the current study highlights the potential interaction of heterophilic antibodies or RF when using sandwich bead ELISA assays. Furthermore, it shows that one cannot simply measure RF and expect interference to be low if RF is low since some sera with low RF titres also showed pronounced interference. Caution should be taken when analysing sera from patients with RA or pSS by sandwich ELISA and assays should be evaluated for its proneness for interaction with heterophilic antibodies/RF.

Study III

Results

Amongst the 51 patients, 47% were ever smokers (8% current smokers, 39% former smokers) (Table 17). Of the 33 controls, 21% were current smokers (Table 17). Amongst controls, sex and age correlated poorly to cytokine levels. Amongst ever smokers at the time of diagnosis, significantly fewer patients had a focal sialadenitis (81 vs 100%; $p = 0.03$) (Table 18). The ESSDAI total score, the ESSPRI total score, IgG, C3, and C4 levels did not significantly differ between ever and never smoking pSS patients (Table 18). Levels of IL-6, IL-12, IL-17 and IL-18 were significantly increased in pSS patients compared to controls, while no major differences between pSS patients and controls for the other cytokines were found (Table 19).

Table 17:
Demographic characteristics of 51 consecutive patients and 33 controls

	Cases	Controls
Age, yrs	61 (52; 69)	47 (39; 61)
Sex, females	49/51 (96)	19/33 (58)
Current/not current smokers,	4/51 (8)	7/33 (21)
Ever/never smokers	24/51 (47)	NA
Fulfilling the AECG for pSS	51/51 (100)	NA
Fulfilling the ACR/EULAR criteria for pSS	51/51 (100)	NA
Disease duration, yrs	12 (6; 18)	NA
Anti-SSA antibody seropositives	40/51 (78)	NA
Anti-SSB antibody seropositives	24/51 (47)	NA
ANA seropositives	40/51 (78)	NA
RF seropositives	26/51 (51)	NA
IgG, g/l	13.0 (10.1; 15.5)	NA
C3, g/l	1.01(0.86; 1.20)	NA
C4, g/l	0.18 (0.13; 0.21)	NA
Lower lip biopsy, focus score ≥ 1 %	37/40 (93)	NA
ESSPRI total score	6 (5; 7)	NA
ESSDAI total score	7 (1; 10)	NA
Nonexocrine symptoms/signs, any of the below %	25/51 (49)	NA
Lymphadenopathy and/or lymphoma ever %	3/51 (6)	NA
Arthritis ever %	4/51 (8)	NA
Cutaneous symptoms ever %	10/51 (20)	NA
Interstitial lung disease ever* %	9/51 (18)	NA
Chronic obstructive lung disease ever %	21/51 (41)	NA
Renal involvement ever %	4/51 (8)	NA
Myositis ever %	0/51 (0)	NA
Peripheral nervous system involvement ever %	1/51 (2)	NA
Raynaud phenomenon ever %	4/51 (8)	NA

pSS: primary Sjögren syndrome; AECG: American-European Consensus Group criteria; ACR: American College of Rheumatology; EULAR: European League Against Rheumatism; ANA: antinuclear antibody; RF: rheumatoid factor; IgG: immunoglobulin G; C3: complement factor 3; C4: complement factor 4; ESSPRI: EULAR Sjögren Patient Reported Index; ESSDAI: EULAR Sjögren Disease Activity Index; EULAR: European League Against Rheumatism; ILD: interstitial lung disease. *Defined as presence of peripheral traction bronchiectasis, honey combing or ground glass opacities.

Table 18:
Comparison of clinical parameters, IgG-levels and complement levels between never smoking and ever smoking pSS patients.

	Ever smokers (pSS) n= 24	Never smokers (pSS) n= 27	P-value⁺
Focal sialadenitis	14/17 (82)	23/23 (100)	0.03*
Anti-SSA- and or SSB-positive	20/24 (83)	20/27 (74)	0.43
ESSDAI	7.5 (1.5; 10)	7 (1; 11)	0.85
ESSPRI	6 (5; 7)	6 (4; 8)	0.68
IgG (g/l)	12.9 (10.1; 17.2)	13.0 (10.1; 15.2)	0.62
C3 (g/l)	1.02 (0.92; 1.22)	0.99 (0.84; 1.16)	0.60
C4 (g/l)	0.19 (0.13; 0.21)	0.16 (0.13; 0.20)	0.40

Values are presented as median (q1-q3) or n/n available (%), *p<0.05, ⁺ Mann-Whitney *U* test

Table 19:
Comparison of cytokine levels between pSS patients and controls

	Cases pg/ml, median (q1-q3) n=51	Controls, pg/ml, median (q1-q3) n=33	P-value⁺
IL-1β	0 (0–0)	0 (0–0)	0.92
IL-2	0 (0–25.0)	0 (0–24.4)	0.86
IL-3	0 (0–67.8)	0 (0–88.7)	0.84
IL-4	0 (0–0)	0 (0–0)	0.20
IL-6	25.2 (14.0–30.9)	15.3 (10.6–22.0)	0.003**
IL-8	19.9 (15.9–22.8)	16.7 (14.1–20.8)	0.06
IL-10	0 (0–0)	0 (0–0)	0.18
IL-12	7.4 (0–10.8)	0 (0–8.3)	0.02*
IL-17	0 (0–51.2)	0 (0–0)	0.002**
IL-18	294 (187.7–500.3)	214.5 (119.0–297.5)	0.008**
IL-33	11.2 (0–16.1)	12.5 (0–17.7)	0.62
IFN-α	0 (0–0)	0 (0–0)	0.53
IFN- γ	0 (0–1.3)	0 (0–1.0)	0.43
TNF-α	12.1 (5.7–16.9)	8.5 (5.9; 11.5)	0.14
BAFF	265.6 (182.4–376.2)	276.1 (142.9; 391.6)	0.79
EGF	139.8 (60.0-227.1)	136.4 (93.2; 177.3)	0.89
Fas ligand	9.5 (7.2–15.3)	11.4 (6.9; 15.2)	0.48
RANTES	15673.7 (11374.1–25397.6)	17073.0 (13345.9; 20037.8)	0.56
TGF-β1	22.4 (6.7–35.6)	12.7 (9.2; 36.6)	0.75

**p*<0.05, ***p*<0.01, ⁺Mann-Whitney *U* test

When comparing ever and never smoking pSS patients, only TNF-α levels were significantly higher in the former group (Table 20). Also, when analysing only anti-SSA positives as well as pSS patients with a shorter than median disease duration amongst pSS patients, a similar lack of association was found. No significant difference was observed in cytokine levels between patients with or without COPD, and ILD, respectively (data not shown).

Table 20:
Comparison of cytokine levels between never smoking and ever smoking pSS patients

	Ever smokers (pSS) n= 24 pg/ml, Median (IQR)	**Never smokers (pSS) n= 27 pg/ml, Median (IQR)**	**P-value+**
IL-1β	0 (0; 0)	0 (0; 0)	0.30
IL-2	0 (0; 28.4)	0 (0; 19.2)	0.85
IL-3	0 (0; 0)	0 (0; 96.9)	0.36
IL-4	0 (0; 0)	0 (0; 0)	1.0
IL-6	25.0 (14.4; 29.4)	25.2 (13.0; 36.7)	0.94
IL-8	21.8 (15.9; 24.1)	18.3 (15.5; 21.4)	0.18
IL-10	0 (0; 0)	0 (0; 39.3)	0.74
IL-12	9.7 (5.6; 12.9)	7.1 (0; 9.8)	0.20
IL-17	0 (0; 75.7)	39.0 (0; 46.6)	0.86
IL-18	364.3 (250.4; 659.3)	234.5 (166.3; 500.3)	0.06
IL-33	11.2 (0; 19.3)	11.2 (0; 15.6)	0.95
IFN-α	0 (0; 0)	0 (0; 0)	0.51
IFN-γ	0.7 (0; 1.3)	0 (0; 1.0)	0.58
TNFα	13.8 (7.2; 20.8)	7.5 (4.9; 15.9)	0.03*
BAFF	256.1 (191.9; 375.2)	265.6 (156.7; 388.3)	0.95
EGF	154.8 (55.4; 252.0)	133.9 (70.8; 202.8)	0.53
FAS ligand	10.3 (7.3; 16.6)	9.2 (6.5; 12.6)	0.50
RANTES	17051.1 (11883.2; 27559.6)	15443.0 (9730.2; 19827.9)	0.46
TGF-β1	21.2 (4.2; 26.9)	22.4 (9.3; 55.0)	0.14

+Mann-Whitney *U* test, *$p<0.05$

In patients with pSS, disease duration was negatively correlated to IL-10 (r=−0.32, p=0.02), IL-12 (r=−0.34, p=0.02) and TNF-α (r = − 0.40, p = 0.004) and positively to TGF-β1 (r = 0.29, p = 0.04). There were no significant correlations between the ESSDAI total score and serum cytokine levels (data not shown). Current smokers entailed only four patients, and therefore statistical analyses were not performed on this group separately.

Discussion

In this study, no significant differences were found in disease activity, as measured by ESSDAI, or by standard laboratory analyses such as IgG levels or complement levels between ever and never smokers. Thus, we did not find any evidence that ever smoking alters the phenotype of pSS. Concerning cytokine levels, only TNFα was significantly higher in the group of ever smokers. This finding should be taken with caution since one would expect other proinflammatory cytokines to be significantly increased if ever smoking were associated with systemic inflammation. Compared to controls increased levels of IL-6, IL-12, IL-17 and IL-18 was found in pSS patients, reminiscent of previous reports regarding IL-6 (173, 227, 228), IL-17 (180), and IL18 (181) in pSS patients. Furthermore, polymorphisms in the IL-12 A gene has been shown to be associated in a genome-wide association study (113). Surprisingly, the levels of BAFF did not differ between patients and controls. BAFF. Elevated levels of BAFF is seen as a hallmark of pSS in many studies (165, 166). A possible explanation can be that the consecutive patients included in the study had a lower disease activity than patients in previous studies or that the in-house made ELISA was not sensitive enough. Problems with analysing BAFF due to posttranslational glycosylations or alternative spliced forms have also previously been reported (229).

Smoking duration varied a great deal between patients (median 21 years, range 1-55 years) (data not shown). Amongst former smokers, median years since smoking cessation was 22 years (range 0–40 years) (data not shown). The heterogenicity in exposure must be taken into consideration since the group with heavier exposure might be too small to discern tap a difference between the groups. As seen in other studies, we found a lower frequency of focal sialadenitis amongst ever smokers (80, 81). A potential explanation of this finding might be a local anti-inflammatory effect by cigarette smoke. Most patients included in the study had a longstanding disease (median 12 years) and disease duration correlated negatively with IL-10, IL-12 and TNF-α and positively with the anti-inflammatory cytokine TGF-β, indicating that the disease develops into a less inflammatory state over time.

The study has some limitations, including the limited sample size and the low number of current smokers amongst the pSS patients. Furthermore, the majority of the patients had long-standing disease, and we cannot exclude that cytokine patterns, and their relation to smoking history, may be different in recently diagnosed patients. The cross-sectional study design is also a limitation. Finally, the control group was not exactly matched as regards sex and age. Strengths of the study include the use of consecutive patients in standard follow-up, likely representative of the general pSS population and the validation of the assays concerning possible interference by rheumatoid factor.

In conclusion, we did not find any evidence that a history of cigarette smoking alters the phenotype of pSS or serum cytokine expression. However, a history of cigarette smoking was associated with a lower frequency of focal sialadenitis. Of note is that most patients had a longstanding disease, the numbers of current smokers were very few and the exposure to cigarette smoke both in time and dose varied a great deal, which may have affected the results.

Study IV

Results

Of 109 consecutive pSS patients, 90 agreed to participate and were included in the study. Median age was 66.5 years (q1-q3: 51.8-73.0), and 93% were females (Table 21). In addition to fulfilling the AECG criteria, all patients also fulfilled the American College of Rheumatology (ACR)/EULAR criteria for pSS.

Smoking habits and IFN signatures of the study population

No patient declared any tobacco smoking apart from cigarette smoking. Six percent of pSS patients were current smokers, whilst 41% and 53% were former and never smokers, respectively. The median time since smoke cessation amongst former smokers was 27 years (q1–q3: 16–39). The median cigarette consumption amongst ever smokers (former + current smokers) was 8.8 pack-years (q1–q3: 4.0–19.0). 72% of pSS patients showed a type I IFN signature. Further characteristics are found in Table 21.

Smoking habits, ESSDAI/ESSPRI and patient characteristics

No significant differences in ESSDAI total score or activity in the ESSDAI domains were found between never, former or current smokers, or between patients with a cigarette consumption as evaluated by pack-years below vs. ≥ 8.8 pack-years, i.e. the median cigarette consumption (Table 22). When comparing ESSPRI sicca domain scores of never (7.0; q1–q3: 5.0–8.0), former (8.0; q1–q3: 5.0–9.0) and current smokers (9.0; q1–q3: 8.5–9.5), there was a statistically non-significant trend of higher values amongst current smokers (p=0.06). Comparing patients with a higher (≥ 8.8 pack-years) and lower (<8.8 pack-years) cigarette consumption, a significantly higher ESSPRI total score [8.0 (q1–q3 6.0–8.3) vs 5.0 (q–q3 3.0–6.3) p=0.01], ESSPRI sicca score [8.0 (q1–q3: 8.0–9.0) vs 6.0 (q1–q3: 5.0–9.0) p=0.05] and ESSPRI pain score [7.0 (q1–q3: 5.0–9.0) vs 4.0 (q1–q3: 2.0–5.0) vs p=0.01] were found in the high consumption group (Table 22). A statistically non-significant trend towards a lower frequency of focal sialadenitis amongst current smokers [3/5

(60%)] compared to former smokers [22/24 (92%)] and never smokers [36/38 (95%)] was found (p=0.06) (Table 22). Amongst former smokers, there were no significant differences in total ESSDAI score, presence of activity in the ESSDAI domains or total ESSPRI score comparing patients below median (27 years) and ≥ median time since smoke cessation (data not shown). No statistically significant differences in type I IFN signature positivity and smoking status were found, although there were numerically fewer type I IFN positive patients amongst current smokers (40%) compared to former (78%) and never (70%) smokers (Table 22). Additionally, no statistically significant differences were found in levels of IgG, C3, C4, presence of anti-SSA, anti-SSB, ANA, or RF when comparing never, former and current smokers (Table 22).

Table 21:
pSS patient characteristics

	n =	
Females (%)		84/90 (93)
Age (years)	90	66.5 (51.8–73.0)
Disease duration	90	13.0 (5.0–21.3)
Current smoker (%)		5/90 (6)
Smoke duration, years	5	48 (36.5–59.5)
Pack–years, years	5	25 (11.3–40.2)
Former smokers (%)		37/90 (41)
Smoke duration, years	37	17 (11.0–30.0)
Pack-years, years	37	8.1 (3.4–17.8)
Time since smoking cessation, years	37	27 (16–39)
Time between smoke cessation and diagnosis, years	37	13 (6.5–21)
Time between smoke cessation and sicca symptoms, years	17	14 (1–21)
Never smoker (%)		48/90 (53)
Ever smokers (current+former smokers) (%)		42/90 (47)
Pack-years, years	42	8.8 (4.0–19.0)
Smoke duration, years	42	19.5 (11.8–35.0)
Years diagnosed with pSS, years	90	13 (5.0–21.3)
Years since first sicca symptom, years	41	15 (5.0–26.0)
Anti-SSA seropositives (%)		78/90 (87)
Anti-SSB seropositives (%)		53/90 (59)
ANA seropositives (%)		70/88 (80)
RF seropositives (%)		62/88 (71)
Focal sialoadenitis (%)		62/68 (91)
IgG, g/L	89	14.8 (+/- 5.35)
C3, g/L	89	0.94 (+/- 0.21)
C4, g/l	89	0.18 (+/- 0.06)
ESSDAI total	89	3 (1.0-7.5)
ESSDAI high activity		7/89 (8)
ESSDAI moderate activity		30/89 (34)
ESSDAI low activity		52/89 (58)
ESSPRI total	89	6.7 (4.7-8.0)
Type I IFN positive		64/89 (72)

Data are presented as *n* cases/*n* total (%). For continuous data: mean (+/- SD)/median (q1–q3). ANA: Anti-nuclear antibodies, C: complement component, ESSDAI: EULAR Sjögren’s syndrome Disease Activity Index, ESSPRI: EULAR Sjögren’s syndrome Patient Reported Index, IFN: interferon, IgG: Immunoglobulin G, RF: rheumatoid factor.

Table 22:
Associations between smoking habits and clinical characteristics in pSS patients.

	Never smokers, n=48	Former smokers, n=37	Current smokers, n=5	p-value	Pack-years < 8.8 years, n=19	Pack-years ≥ 8.8 years, n=23	p-value
ESSDAI total	3 (1.0–8.0)	4 (1.0–7.5)	1.0 (0.0–8.5)	0.55	4 (1.0–7.0)	4.0 (0.0–9.0)	0.87
ESSDAI activity in each domain, *n* (%)							
Constitutional	6/47 (13)	7/37 (19)	0/5 (0)	0.61	3/19 (16)	4/23 (17)	1.0
Lymphadenopathy	3/47 (6)	1/37 (3)	0/5 (0)	0.71	1/19 (5)	0/23 (0)	0.45
Glandular	5/47 (11)	5/37 (14)	0/5 (0)	0.86	1/19 (5)	4/23 (17)	0.36
Articular	4/47 (9)	4/37 (11)	2/5 (40)	0.12	4/19 (21)	2/23 (9)	0.38
Cutaneous	3/47 (6)	3/37 (8)	0/5 (0)	1.0	1/19 (5)	2/23 (9)	1.0
Pulmonary	5/47 (11)	6/37 (16)	1/5 (20)	0.51	4/19 (21)	3/23 (13)	0.68
Renal	4/47 (9)	4/37 (11)	0/5 (0)	0.83	0/19 (0)	4/23 (17)	0.11
Muscular	0/47 (0)	1/37 (3)	0/5 (0)	0.47	0/19 (0)	1/23 (4)	1.0
PNS	2/47 (4)	2/37 (5)	0/5 (0)	1.0	1/19 (5)	1/23 (4)	1.0
CNS	2/47 (4)	0/37 (0)	0/5 (0)	0.56	0/19 (0)	0/23 (0)	-
Haematological	11/47 (23)	8/37 (22)	0/5 (0)	0.76	5/19 (26)	3/23 (13)	0.43
Biological	25/47 (53)	20/37 (54)	2/5 (40)	0.89	10/19 (53)	12/23 (52)	1.0
ESSPRI total score	6.7 (4.7–7.7)	6.0 (4.5–8.0)	8.0 (6.2–8.7)	0.24	5.0 (3.0–6.3)	8.0 (6.0–8.3)	0.01*
ESSPRI domain scores							
Sicca	7.0 (5.0–8.0)	8.0 (5.0–9.0)	9.0 (8.5; 9.5)	0.06	6.0 (5.0–9.0)	8.0 (8.0–9.0)	0.05*
Fatigue	7.0 (5.0–8.0)	7.0 (3.0–8.0)	8.0 (4.0; 9.0)	0.64	5.0 (2.0–8.0)	8.0 (5.0–9.0)	0.09
Pain	5.0 (1.0– 7.0)	5.0 (3.0; 8.0)	8.0 (3.5; 9.5)	0.18	4.0 (2.0–5.0)	7.0 (5.0–9.0)	0.01*
Anti-SSA seropositive (%)	40/47 (85)	32/37 (87)	5/5 (100)	1.00	18/19 (95)	19/23 (83)	0.36
Anti-SSB seropositives (%)	28/47 (60)	22/37 (60)	2/5 (40)	0.78	13/19 (68)	11/23 (48)	0.22
ANA seropositives (%)	38/46 (83)	28/36 (78)	4/5 (80)	0.92	13/19 (68)	19/22 (86)	0.26
RF seropositives (%)	33/46 (72)	25/36 (69)	3/5 (60)	0.89	12/19 (63)	16/22 (73)	0.51
Focal sialoadenitis (%)	36/38 (95)	22/24 (92)	3/5 (60)	0.06	10/12 (83)	15/17 (88)	1.0
IgG (g/L)	14.4 (+/- 5.09)	15.7 (+/- 5.7)	12.8 (+/- 4.99)	0.40	15.9 (+/- 6.98)	14.9 (+/- 4.50)	0.68
C3 (g/L)	0.92 (+/- 0.19)	0.94 (+/-0.23)	1.04 (+/- 0.29)	0.43	0.94 (+/- 0.25)	0.97 (+/- 0.24)	0.73
C4 (g/L)	0.18 (+/- 0.06)	0.18 (+/- 0.06)	0.20 (+/- 0.07)	0.67	0.19 (+/- 0.08)	0.19 (+/- 0.05)	0.98
Type I IFN positive (%)	33/47 (70)	29/37 (78)	2/5 (40)	0.16	15/19 (79)	16/23 (70)	0.73

*= p<0.05; Data are presented as n/n total (%). For continuous data: mean (+/- SD)/median (q1–q2). Comparisons between the mean or median of more than 2 groups were performed using One-way ANOVA or Kruskal-Wallis test. Means or medians of two groups were compared using independent T-test or Mann Whitney U-test. Discrete variables were compared using Chi^2 test or Fisher's exact test. ESSDAI is presented as ESSDAI total score as well as presence of activity in each ESSDAI domain. ESSPRI is presented as ESSPRI total as well as domain scores. ANA: Anti-nuclear antibodies, C: complement component, CNS: central nervous system, ESSDAI: EULAR Sjögren's syndrome Disease Activity Index, ESSPRI: EULAR Sjögren's syndrome Patient Reported Index, IFN: interferon, IgG: Immunoglobulin G, PNS: peripheral nervous system, RF: rheumatoid factor.

IFN signature, ESSDAI/ESSPRI and patient characteristics

When studying associations between type I IFN signature and ESSDAI scores, only the presence of activity in the ESSDAI articular domain was significantly lower in the type I IFN signature positive group in comparison with the type I IFN signature negative group (0.0 (0.0; 0.0) vs 0.0 (0.0; 2.0), $p<0.01$). In addition, the ESSPRI total score (6.0 (4.0; 7.7) vs 7.7 (5.2; 8.2); $p=0.04$), ESSPRI sicca score (7.0 (5.0; 8.0) vs 8.0 (6.0; 9.0); $p=0.03$) and ESSPRI pain score (4.5 (1.3; 7.0) vs 6.0 (5.0; 8.0); $p= 0.02$) were also significantly lower in the type I IFN positive group (Table 3). Type I IFN signature positive patients were significantly more often seropositive for anti-SSA (97% vs 60%; $p<0.01$), anti-SSB (69% vs 32%; $p<0.01$), ANA (89% vs 58%; $p<0.01$), RF (81% vs 42%; $p<0.01$), and had higher titers of IgG (mean 15.7 g/L; SD 5.26 vs 12.5 g/L; SD 4.95, $p=0.01$) compared to type I IFN signature negative patients. However, no differences in the presence of focal sialoadenitis, or levels of complement 3 or 4 were found between these groups (Table 23). No association between disease duration and type I IFN positivity was found.

Table 23:
Clinical characteristics stratified on IFN activation

	IFN I neg, n=25	IFN I pos, n=64	p-value
ESSDAI total score	3.0 (0.0–8.0)	3.0 (1.0–7.8)	0.85
ESSDAI activity in each domain			
Constitutional	5/25 (20)	8/64 (13)	0.50
Lymphadenopathy	1/25 (4)	3/64 (5)	1.0
Glandular	2/25 (8)	8/64 (13)	0.72
Articular	7/25 (28)	3/64 (5)	<0.01*
Cutaneous	2/25 (8)	4/64 (6)	1.0
Pulmonary	1/25 (4)	11/64 (17)	0.17
Renal	2/25 (8)	6 /64 (9)	1.0
Muscular	0/25 (0)	1/64 (2)	1.0
PNS	1/25 (4)	3/64 (5)	1.0
CNS	1/25 (4)	1/64 (2)	0.49
Haematological	4/25 (16)	15/64 (23)	0.44
Biological	13/25 (52)	34/64 (53)	0.92
ESSPRI total score	7.7 (5.2–8.2)	6.0 (4.0–7.7)	0.04*
ESSPRI domain scores			
Sicca	8.0 (6.0–9.0)	7.0 (5.0–8.0)	0.03*
Fatigue	8.0 (5.0–9.0)	7.0 (5.0–8.0)	0.26
Pain	6.0 (5.0–8.0)	4.5 (1.3–7.0)	0.02*
Anti-SSA seropositives	15/25 (60)	62/64 (97)	<0.01*
Anti-SSB seropositives	8/25 (32)	44/64 (69)	<0.01*
ANA seropositives	14/24 (58)	56/63 (89)	<0.01*
RF seropositives	10/24 (42)	51/63 (81)	<0.01*
Focal sialoadenitis	16/18 (89)	45/49 (92)	0.66
IgG (g/L)	12.5 (+/- 4.95)	15.7 (+/- 5.26)	0.01*
C3 (g/L)	0.94 (+/- 0.21)	0.93 (+/- 0.22)	0.83
C4 (g/L)	0.19 (+/- 0.07)	0.18 (+/- 0.06)	0.42

*p<0.05; Data are presented as n/n total (%). For continuous data: mean (+/- SD)/median (q1–q3). Independent T-test for continuous, normally distributed variables, Mann Whitney U-test for continuous non-normally distributed variables, Chi2 test or Fisher's exact test for discrete variables ESSDAI is presented as an ESSDAI total scores as well as presence of activity in each ESSDAI domain. ESSPRI is presented as ESSPRI total as well as domain scores. ANA: Anti-nuclear antibodies, C: complement component, CNS: central nervous system, ESSDAI: EULAR Sjögren's syndrome Disease Activity Index, ESSPRI: EULAR Sjögren's syndrome Patient Reported Index, IFN: interferon, IgG: Immunoglobulin G, PNS: peripheral nervous system, RF: rheumatoid factor.

Discussion

In this study, 6%, 41% and 53% of patients were current, former and never smokers respectively. Seventy-two percent of the patients were type I IFN positive, which is in line with a previous study that found that 81% of pSS patients in a UK cohort and 53% of pSS patients in a cohort from the Netherlands were type I IFN positive when assessing expression of the same set of genes included in this study. This indicates that the patient sample and type I IFN analysis was adequate. No associations were found in ESSDAI total score or activity in ESSDAI domains, by smoking habits or cigarette consumption. Concerning symptoms, as measured by ESSPRI, a higher cigarette consumption was associated with higher scores in ESSPRI total score and ESSPRI sicca and pain domains. No significant associations between type I IFN positivity and smoking habits was found. The number of current smokers in the present study was found to be very low (n=5), making conclusions about the effect of current smoking difficult. However, we noted that a type I IFN signature was only found in two out of five (40%) of current smokers compared to 70% of never smokers and 78% of former smokers. This could be interesting to investigate further in a study including more current smokers, although it would require a large group of patients considering the low number of current smokers in this present group of patients. Furthermore, comparing type I IFN signature positive and negative patients, the former had significantly *lower* values in the ESSPRI total score as well as the ESSPRI sicca and pain domain scores. On the other hand, when comparing current smokers to not current smokers (former+never smokers), current smokers had significantly *higher* values in the ESSPRI sicca domain and, although only numerically, in the ESSPRI pain domain (data not shown). Since the differences between current and not current smokers and type I IFN signature positive and negative patients are similar, although reverse, one could speculate whether current smoking inhibits the effect of IFN I-signalling, as cigarette smoking has previously been shown to affect IFN I signalling (93). Again, this is speculative since the low numbers of smokers in this study does not allow us to draw any firm conclusions.

Concerning symptoms, we found an inverse association between type I IFN signature and ESSPRI total, sicca and pain scores. A negative association between ESSPRI and IFN signature and other proinflammatory cytokines has previously been described (48, 170). A possible explanation for this finding could be that the mechanisms regulating inflammation also affect symptoms such as fatigue and pain as proposed by Howard Tripp et al (170). There are also reports that IFNα inhibits nociceptive transmission in the spinal cord, which could provide an explanation to the negative association between type I IFN signature and the ESSDAI pain domain (230). Furthermore, there are also reports that treatment with low dose IFNα improves salivary flow in pSS patients (161), which could be due to an increased expression of the aquaporine-5 gene (162).

Smoking exposure in this cohort of consecutive patients was mostly long before the study started and the amount of consumed cigarettes varied a great deal. Therefore, the population of former smokers was heterogenous. As expected, the number of current smokers was low, making it difficult to draw conclusions about the association with current smoking. Some trends amongst current smokers were noted though, which could be further investigated in a larger study including more current smokers. However, there is a trend towards reduced frequency of daily smokers in the general population (231). Therefore, it might be difficult to include a larger number of current daily smokers in future studies.

Strengths of the current study include the use of consecutive, well-characterised patients in routine clinical care. However, there were several limitations of the study, including the small number of current smokers and heterogenicity concerning cigarette consumption. Retrospectively acquired data on smoking also has limitations due to recall bias. Potential confounders of sicca symptoms, e.g. use of antidepressants or other drugs with anti-cholinergic side effects, were not available. Most patients had long-standing pSS, and we cannot exclude that different patterns may be observed in patients with recent onset of clinical disease.

No significant differences were found between never, former or current smokers in ESSDAI total score or presence of activity in the ESSDAI domains. Of note is that current smokers were few in this study making it difficult to draw any firm conclusions regarding this group. Likewise, no difference in type I IFN signature was found by smoking habits or smoking exposure (pack-years). However, patients with a higher cumulative smoking exposure scored significantly higher in ESSPRI total, and ESSPRI sicca and pain domains scores whilst type I IFN signature positive patients scored significantly lower in ESSPRI total, ESSPRI sicca and pain domains and ESSDAI articular domain. In conclusion, there does not seem to be a strong and consistent association between former smoking and disease activity, patient reported symptoms and laboratory signs of systemic inflammation in pSS, at least not in established disease.

General discussion

The aims of this thesis were to investigate potential epidemiological risk factors and potential biomarkers for pSS. We approached these aims by firstly trying to identify epidemiological risk factors for pSS, where cigarette smoking was identified as one, and after that, to see whether the risk factor was associated with biomarkers (cytokine profile and activation type I IFN genes) as well as disease activity and disease phenotype.

Studies of epidemiological risk factors for the development of pSS are few and, apart from the fact that pSS mainly affects women, information regarding risk factors is scarce. One aim of this thesis was to add to the knowledge about risk factors for the development of pSS. Since the population of pSS patients previously included in the health studies (MPMP and MDCS) were few, we could not use the full potential of these population-based surveys as we had to select identical variables in the health studies and the Malmö Sjögren's syndrome Registry or variables that could be translated into a common variable. Nevertheless, we could extract pre-diagnostical information about smoking, level of education and socioeconomic status which, to the best of our knowledge, has never been done before. Although level of education and socioeconomic status may be surrogate markers of other risk factors associated to the groups, such as environmental exposures associated with certain occupations, diet, problem-solving capacities, and others, the possible association to disease development still is of interest. Previous studies in RA, using the same health surveys, did find an impact of low formal education on disease development (104), confirming results from other studies in RA (223, 232, 233). In fact, many chronic diseases are found more frequently in individuals with less than 12 years of formal education (223) which is why it was of interest to see whether this was true for pSS as well. However, we did not find an impact on the development of pSS by level of education or by socioeconomic status, although a potential bias might be that patients with a lower formal education or of a lower socioeconomic status might not have sought medical attention for their symptoms and might not have been diagnosed with pSS. Concerning the pre-diagnostical information on smoking, this is of specific interest in pSS, since the development of the disease is often insidious and both patient's and doctor's delay is substantial. The nested case-control design of the study reasonably acquires more reliable data than cross-sectional studies since it limits recall-bias. Interestingly, although the data on smoking was acquired in a median 8.2 years before diagnosis, there was a lower prevalence of current smokers, reminiscent of results from other studies with a cross-sectional design (75, 80). Although this is no formal proof that smoking affects the development of pSS, it weakens the hypothesis that the lower frequency of smokers amongst pSS patients is only due to the sicca symptom. The larger study from the United States by Stone et al. (80), referred to earlier, also supports the notion that the reason for the lower prevalence of smoking is not only sicca symptoms. The control group in that study consisted of non-pSS sicca patients and nevertheless a lower frequency of current smokers was found in pSS patients. It is also important to note that both a focal sialadenitis and the presence of anti-SSA antibodies are reported in lower frequencies amongst current smokers (80, 81). Since the presence of either a focal sialadenitis or anti-SSA is mandatory for the diagnosis of pSS in accordance with the AECG or ACR/EULAR criteria, this finding results in smoking pSS patients not being classified as pSS, as has previously been highlighted by others (234). On the other hand, longstanding smoking has also

been shown to reduce salivation (235, 236) and this might potentially affect the classification, at least the AECG criteria, “in favour” of smoking individuals.

Study II illustrates the potential problems RF can cause when using sandwich ELISA for analysing cytokine levels and it is important to keep this in mind. It is advisable to check the individual ELISA used in studies for its proneness to interact with heterophilic antibodies/RF by, for instance using non-sense ELISAs (where no signal should be detected and thus, if signal is detected, it is an evidence of cross-binding by RF or heterophilic antibodies) or adding a blocker to see if it changes the measured cytokine levels. Using Fab fragments instead of whole IgG antibodies as capture and detection antibodies can also reduce the problem (237).

The known effect of smoking on the immune system and on other autoimmune diseases also makes it plausible that smoking can affect the development of pSS. A comprehensive review of the topic is beyond the scope of this thesis, but some examples were presented previously in the introduction. The interest in whether smoking does affect development of pSS or not is not so as to know which recommendations to give patients, or their relatives, concerning smoking. Smoking has profound negative effects on health and everyone should be recommended, and helped, to quit smoking. The interest is in whether this can help us to understand how pSS develops. Inflammatory bowel disease can serve as an example where the differences in pathogenesis between ulcerative colitis and Crohn’s disease may be the cause of their different associations to smoking. Ulcerative colitis is ameliorated and Crohn’s disease worsened by smoking. Furthermore, Crohn’s disease is considered a Th1-driven disease (where IFN-γ and IL-12 play important roles) whilst ulcerative colitis is considered a Th2-driven disease (where IL-4 and IL-5 are important cytokines). Also, in Crohn’s disease there are reports of a higher prevalence of ileal disease and a lower frequency of colonic involvement in smokers (238) Thus, both the type of inflammation and the constitution of the target organ may be important for the effect of smoking. Two of the studies included in this thesis investigated pSS phenotype, by assessment of organ involvement, as well as cytokine profiles of the patients (Study III and IV) without finding any major differences between the groups other than a lower frequency of focal sialadenitis amongst current smokers (Study III) whilst no association with disease activity as measured by ESSDAI (Study III and V). Thus, Study III and IV do not support the notion that smoking alters the risk of systemic complications in pSS. On the other hand, pSS patients with a higher exposure to smoking did have more symptoms of dryness and pain, although this might have other explanations besides smoking having a potential effect on the activity of pSS (235, 239).

The experiences from other rheumatological diseases, such as RA, PsA, and SpA, treated with cytokine inhibitors encourage the search for key cytokines that potentially could be treatment targets in pSS. Several studies have found differently

expressed cytokines, both in serum and in biopsy specimens, between pSS patients and controls. Despite this, clinical trials using e.g. anti-TNF have been disappointing. There have been similar problems in clinical trials on SLE, a disease pathologically more similar to pSS, which might be due to the systemic nature of these diseases and issues about the instruments of evaluation of treatment effects. Also, in both pSS and SLE there can be difficulties in assessing what outcomes are due to disease activity/inflammation as opposed to those due to damage. In addition, the length of some studies might have been too short, as a follow-up study on anti-BAFF treatment in pSS suggests (204), showing that actively treated patients did not worsen in the same manner as non-treated patients. Although the ESSDAI is the only validated index for measuring disease activity, there are some important issues to consider. Cross-sectional studies analysing the association between cytokine expression, including IFN signature, and ESSDAI have also been inconsistent (48, 157, 240). Also, in general only 1/3 of patients have extraglandular manifestations and thus 2/3 of pSS patients score 0. Accordingly, 21 % of pSS patients scored 0 in Study IV. In summary, although ESSDAI is currently the best alternative measuring disease activity, there are questions about its sensitivity and sensitivity to change which potentially could explain the poor associations between former smoking and phenotype in the studies included in this thesis.

There are some important points to consider regarding the results of Study III and IV: 1. Generally, the number of patients were few in the studies, reducing the power of detecting true differences (type I error). 2. Of note is that larger numbers of current smokers were lacking, which is why we cannot draw any firm conclusions regarding the effect of current smoking. 3. Finally, there was a heterogenicity concerning cigarette consumption and years since smoke cessation amongst former smokers which might also blur the results.

When studying pSS, there are some general challenges. pSS is generally a low inflammatory disease with insidious onset, making early diagnosis hard. This is a problem since patients may present evidence of irreversible damage in their exocrine glands already at diagnosis, and with a longstanding inflammatory process that has been going on for years, the inflammatory process may also be harder to treat. Furthermore, there is a heterogenicity within the group of pSS patients where some patients have lupus-like systemic features whilst others have a low inflammatory disease with sicca symptoms and features more similar to fibromyalgia with pain and fatigue. Of note is that one study has found that fatigue is actually negatively associated with proinflammatory cytokines (170). Therefore, a stratification of patients based on both patient symptoms and laboratory results (ACR abstract number 3031 2016) has been proposed. The diagnostic criteria have been discussed for decades and recently a new criteria set has been adopted (the ACR/EULAR criteria) (29), enabling earlier diagnosis of the disease at least in a subset of patients. Although this is an improvement, the obvious question is whether

it would be possible to identify patients before they develop severe dry mouth or before they develop measurable anti-SSA antibodies? The answer is most probably “no” or at least “not yet”. The focus to date must be to identify highly active patients for clinical trials. However, the new ACR/EULAR criteria set is a step in the right direction as they allow for a patient being classified with pSS not only if having sicca symptoms but also if experiencing pSS typical systemic symptoms in combination with a focal sialadenitis and presence of anti-SS-A antibodies, even if sicca symptoms are lacking or have not yet developed.

Conclusions

- A negative association between cigarette smoking a subsequent pSS diagnosis was found
- There were no associations between socioeconomic status or educational level and a subsequent pSS diagnosis
- Rheumatoid factor can cause erroneous results when using bead ELISA, the level of interference is not linear to the level of measured of RF and the problem can be reduced using blockers of heterophilic antibodies/rheumatoid factor.
- Former smoking does not affect disease activity of pSS as measured by ESSDAI
- A higher cumulative cigarette consumption is associated with more dryness and pain symptoms as measured by ESSPRI
- Former smoking is not associated to different expression of cytokines or type I IFN signature amongst pSS patients
- No associations between ESSDAI total score and measured levels of cytokines or presence of an type I IFN signature were found despite that 72% of the patients in Study IV had evidence of an activated type I IFN system.

Future perspectives

I would briefly like to mention some possible future perspectives

- The international efforts to create larger cohorts enabling stratification of patients and higher statistical precision should continue. This would for instance enable studying the effect of current smoking on disease activity.
- Further investigation of the immunomodulatory effect of nicotine and other compounds in cigarette smoke since this could possibly identify new potential treatment targets.
- Continuation of the work of finding biomarkers for pSS enabling early diagnosis and monitoring of the disease
- Further development of the clinical instruments for assessment of disease activity
- Gathering of information from sicca syndrome induced by check-point inhibitors and what lessons can be learned
- Further investigation of the interplay between the immune system and viral infections.

Populärvetenskaplig sammanfattning

Primärt Sjögrens syndrom är en autoimmun sjukdom som drabbar så kallade exokrina körtlar, d.v.s. de körtlar som fuktar slemhinnor. Framför allt drabbas spottkörtlar och tårkörtlar med svår torrhet i mun och ögon som följd. Svår karies är vanligt till följd av muntorrheten. Salivkörtlarna kan av inflammationen bli förstorade och ömmande. Förutom torrheten besväras patienterna ofta av en abnorm trötthet, och värk i kroppen är vanligt förekommande hos dessa patienter. Hos ca 1/3 av patienterna kan även andra organ, så som lungor, leder, hud, njurar och nerver drabbas. Sjukdomen förekommer i alla åldrar och hos både kvinnor och män men drabbar framför allt kvinnor (kvinnor:män 14:1) och främst efter klimakteriet. Oftast har sjukdomen en smygande debut. Man uppskattar att sjukdomen drabbar ca 1-9/10.000. I dagsläget finns ingen godkänd behandling förutom medel som stimulerar den kvarvarande funktionen i körtlarna. Vid svårare tillstånd används dock immunhämmande medel enligt de erfarenheter man har från andra autoimmuna sjukdomar. Orsaken till sjukdomen är okänd men den inflammation man ser är lik den inflammation kroppen reagerar med vid virusinfektioner. Att virus skulle spela en roll för uppkomsten eller skulle orsaka sjukdomen har man dock ej kunnat visa. Det finns endast få studier där man undersökt vilka miljöfaktorer som spelar roll för utvecklingen av primärt Sjögrens syndrom och man har inte funnit andra orsaker än att kön och ärftlighet spelar in samt att färre patienter med primärt Sjögrens syndrom röker jämfört med kontroller. Studier av miljöfaktorer, så kallade epidemiologiska studier, är viktiga vägvisare för att förstå hur sjukdomar utvecklas. Med termen ”biomarkör” menas att man mäter något, t.ex. ett protein i blodet, som följer sjukdomsaktiviteten och därmed kan användas när man behandlar eller diagnostiserar en sjukdom. En biomarkör kan ha låg eller hög specificitet. Exempelvis kan en hög sänka visa att man har en inflammation i kroppen men inte vad som orsakar inflammationen medan en analys av hepatit C RNA med hög sannolikhet säga att man har hepatit C.

Det första arbetet i avhandlingen är ett epidemiologiskt arbete där data från två stora hälsoundersökningar används. I studierna Malmö Förebyggande Medicin och Malmö Kost Cancer inbjöds en stor del av befolkningen inom ett visst åldersspann i Malmö att delta. I studierna fick man fylla i relativt omfattande formulär med frågor om hälsa, medicinering, alkohol, arbete, rökning mm. Man registrerade även t.ex. längd och vikt samt tog blodprover som lagrats sedan dess. Efter ett antal år

kommer vissa individer att utveckla sjukdom medan andra inte och man kan då analysera om något skiljde de som utvecklat sjukdom från de som inte gjort det utifrån frågeformulären respektive blodproven. Målet är att kunna hitta påverkningsbara riskfaktorer för sjukdom. I den första studien fann vi att personer som senare diagnosticerats med primärt Sjögrens syndrom inte hade ett pågående cigarettbruk, när befolkningsstudien gjordes, i samma utsträckning som de som ej utvecklat sjukdomen. Frågorna hade då besvarats i snitt (median) 8.2 år före diagnos av primärt Sjögrens syndrom. Fyndet att rökning är kopplat till en mindre risk att utveckla sjukdomen stämmer överens med tidigare tvärsnittsstudier där man sett att patienter med pSS röker i mindre utsträckning än kontroller. Torrhet i luftrör, munhåla och ögon ökar irritationen av cigarettrök och kan vara en orsak till att man slutar röka i högre grad om man har pSS. I och med att vår studie visar att denna koppling finns många år innan diagnos av pSS talar det för att det inte bara är torrheten i mun och luftrör som gör att man slutar röka. Det finns andra exempel på sjukdomar som kan förvärras av rökstopp vilket beror på att röken även påverkar immunsystemet. En bidragande orsak till den förhöjda risken för flera olika cancrar vid rökning kan också vara på att immunsystemet får svårare att slå ut celler med skador i sitt DNA innan de utvecklas till cancer p.g.a. immunhämning.

Patienter med primärt Sjögrens syndrom har ofta en viss typ av antikroppar i blodet som binder till andra antikroppar (så kallad reumatoid faktor, RF). När man i laboratorier ska mäta olika proteiner i blodet använder man sig ofta av en teknik där märkta antikroppar binder till proteinet man ska mäta. (ELISA: Enzyme-Linked Immunosorbent Assay) Det finns en risk att RF binder till dessa antikroppar och stör analysen. I andra arbetet undersökte vi hur en sådan analys påverkades när man analyserade serum från patienter (både med ledgångsreumatism och primärt Sjögren syndrom) som hade RF i blodet. Resultaten visade att man fick kraftiga felvärden om man inte tillsatte en speciell blockare, som binder upp RF, till proverna.

I den tredje studien följde vi upp resultaten från den epidemiologiska studien. I och med att vi funnit att rökning inte var lika vanligt hos patienter med primärt Sjögrens syndrom ville vi se huruvida nuvarande eller tidigare rökning påverkade allvarlighetsgraden eller sjukdomsuttrycket hos patienter med primärt Sjögrens syndrom. Detta gjordes genom att mäta flera olika signalproteiner (cytokiner) i blodet hos patienter samt hos kontroller. I denna studie kontrollerade vi att RF inte påverkade analysen baserat på de resultat vi fick i den andra studien. Aktiviteten i patienternas sjukdom hade också registrerats enligt ett speciellt skattningsformulär (ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index), där man skattar inflammationen i flera olika organsystem. Patienternas symtom i form av torrhet, trötthet och smärta skattades med hjälp av ett annat formulär: ESSPRI (EULAR Sjögren's Syndrome Patient Reported Index). I denna studie fann vi inga skillnader mellan de som aldrig rökt och de som någon gång rökt regelbundet. Endast fyra

patienter var nuvarande rökare av de 51 patienter som deltog i studien varför man inte kunde uttala sig om huruvida pågående rökning påverkar sjukdomsaktiviteten.

I den fjärde studien gjorde vi en liknande analys men med ett något större antal patienter, 90 st. Vid primärt Sjögrens syndrom har man funnit att det så kallade typ I interferonsystemet är aktiverat hos en stor andel av patienterna. Man har även funnit att typ I interferonsystemet är aktiverat vid andra autoimmuna sjukdomar. Typ I interferoner är signalämnen som är viktiga för virusförsvaret och som reglerar ett stort antal gener. Genom att mäta uttrycket av ett antal gener kan man få en uppfattning om denna del av immunförsvaret är aktivt. I den fjärde studien använde vi oss av denna teknik och mätte även sjukdomsaktivitet enlig samma skattningsinstrument som i den tredje studien. Vi fann att över 70% av patienterna i studien hade ett aktiverat interferonsystem, vilket är i linje med tidigare studier. Vi fann dock inte någon skillnad mellan icke rökare och tidigare rökare vare sig vad gäller uttrycket av interferonreglerade gener eller vad gäller sjukdomsaktivitet. På samma sätt som i den tredje studien var det få pågående rökare bland patienterna och vi kunde därmed inte uttala oss om effekten av pågående rökning. Vi fann dock att de med högre total konsumtion av cigaretter hade mer symtom i form av torrhet och smärta samt att de patienter som inte hade ett aktiverat interferonsystem hade mer torrhet och smärta. Vi fann dock ingen statistisk koppling mellan rökning och uttrycket av interferonreglerade gener.

Sammanfattningsvis fann vi att rökning var kopplat till en lägre risk att utveckla primärt Sjögrens syndrom. Vi kunde dock ej finna några bevis för att tidigare rökning påverkar allvarlighetsgraden av sjukdomen vare sig när man mäter sjukdomsaktiviteten med skattningsinstrumentet ESSDAI, när vi mätte inflammatoriska signalämnen i blodet eller uttrycket av interferonreglerade gener. Dock fann vi att en högre total konsumtion av cigaretter (kumulativ konsumtion) gav mer symtom i form av ökade besvär med torrhet och smärta. Vidare bör man vara observant när man analyserar serum från patienter med RF och kontrollera att den metod man använder sig av inte ger felaktiga resultat orsakade av att RF stör analysen.

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Study I

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Arthritis Research & Therapy

Cigarette smoking and the risk of primary Sjögren's syndrome: a nested case control study

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Abstract

Background: Smoking is reported to affect the risk of a number of chronic disorders, including rheumatic diseases. Previous cross-sectional studies have shown a lower frequency of smoking in patients with primary Sjögren's syndrome (pSS). The aim of this study was to investigate the impact of smoking and socioeconomic status on the risk of subsequent diagnosis of pSS in a nested case-control study.

Method: Participants in two large population-based health surveys who were later diagnosed with pSS were identified through linkage with the Malmö Sjögren's Syndrome Register. Matched controls were obtained from the health surveys.

Results: Sixty-three patients with pSS with pre-diagnostic data from the health surveys were identified. Current smoking was associated with a significantly lower risk of later being diagnosed with pSS (odds ratio (OR) 0.3; 95% CI 0.1–0.6). Furthermore, former smoking was associated with an increased risk of subsequent pSS diagnosis (OR 4.0; 95% CI 1.8–8.8) compared to never smoking. Similar results were found in a sub-analysis of patients with reported symptom onset after inclusion in the health surveys. Socioeconomic status and levels of formal education had no significant impact on the risk of later being diagnosed with pSS.

Conclusion: In this nested case-control study, current smoking was associated with a reduced risk of subsequent diagnosis of pSS. In addition, former smoking was associated with an increased risk. Whether this reflects a biological effect of cigarette smoking or other mechanisms should be further investigated in future studies.

Keywords: Primary Sjögren's syndrome, Epidemiology, Smoking, Socio-economy, Risk factor

Background

Primary Sjögren's syndrome (pSS) is an autoimmune disease primarily affecting the salivary and lacrimal glands, causing oral and ocular dryness. Other organs such as lungs, kidneys, nervous system, and skin may also be affected in the disease [1]. pSS is associated with B-cell activation, presence of various autoantibodies, systemic symptoms and an increased risk of non-Hodgkin lymphoma [2, 3]. The onset of the disease is often insidious and development of autoantibodies is seen years before onset of clinical disease [4, 5].

The estimated prevalence varies widely. When using the American European consensus group criteria (AECG criteria) [6] the prevalence of pSS is estimated to be between 11.3 and 236.1/100,000 in different European populations. Annual incidence estimates vary between 3.1 and 5.3/100000 [7].

The aetiopathogenesis of pSS is poorly understood but it entails genetic factors [8], hormonal factors [9] and possible viral infections [10], while environmental triggers are less well-studied. Smoking is a widely known risk factor for several malignant diseases [11, 12], but also for chronic inflammatory disorders such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Crohn's disease [13, 14]. Retrospective studies and studies with exposure measured before diagnosis [15–17] in RA indicate that smoking is particularly associated with seropositive RA. In some diseases, such as ulcerative colitis, Behcet's disease

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and Parkinson's disease, smoking seems to have a protective effect [18–20]. Only three studies have specifically addressed the association between smoking and pSS and in none of them has smoking been assessed prior to pSS diagnosis [21–23].

The closeness of cigarette smoke to mucosal membranes and exocrine glands, and the effect of the cigarette smoke on the immune system, makes the influence of cigarette smoking on the development of pSS interesting. However, when studying associations with smoking, social status has to be taken into consideration because it can correlate with both smoking and other exposures such as environmental toxins and dietary factors. Finding epidemiological risk factors for pSS might help us understand the underlying pathogenesis and thus guide further studies. As smoking affects the development of several diseases, including rheumatic disease, we hypothesized that smoking also might affect the development of pSS. To our knowledge, this is the first nested case-control study on the relationship between smoking and socioeconomic status and subsequent diagnosis of pSS.

Method

Aim

The aim of this study was to investigate the impact of smoking and socioeconomic status on the risk of later being diagnosed with pSS.

Source populations

This nested case-control study used information from the Malmö Preventive Medicine Project (MPMP) and the Malmö Diet and Cancer Study (MDCS). The MPMP is a health survey performed in Malmö, Sweden (current population approximately 320,000, at the time of the survey 235,000) between 1974 and 1991 entailing 33,346 individuals. Of these, 22,444 were men born between 1921 and 1938 and 10,902 were women born between 1925 and 1938. The main purpose of the MPMP was to study risk factors for cardiovascular disease and alcohol abuse. Details on the recruitment have been described previously [24]. The overall participation rate was 71%.

The MDCS is a health survey performed in Malmö between 1991 and 1996 that included 30,447 individuals born between 1923 and 1945. The total source population was 74,138, and the participation rate was 41%. The survey has been described in detail previously [24, 25]. The main purpose of the MDCS was to study risk factors for cancer.

Exposure information

Participants in the health surveys filled out self-administered questionnaires on personal and medical information, including smoking and level of education. After merging data from the MPMP and the MDCS, cases and controls were classified as current/not current smokers at the time of participation in the health survey, and also as current/former/never smokers. In the MDCS, formal education was classified as follows: ≤8 years of elementary school, 9–10 years, 11–12 years, >12 years and university degree. In the MPMP, information was available on whether the individual had attended elementary school, secondary school or higher education. A low level of formal education was classified in the MDCS as ≤8 years and in the MPMP as elementary school only.

Information on socioeconomic status had previously been incorporated in MPMP and MDCS by linking the databases with data on standardized socioeconomic status by occupation, based on information from Statistics Sweden, as previously described in detail [26]. The categorization of socioeconomic status by Statistics Sweden includes a division in groups corresponding to working class, middle class, and a group of self-employed inhabitants. For the present study, participants were classified as blue-collar workers or white-collar workers. Blue-collar workers included both skilled and unskilled manual workers, corresponding to the working-class group from Statistics Sweden. White-collar workers included non-manual employees of high level (occupations normally requiring six years of post-comprehensive school education, e.g. higher civil servants and executives), medium level (occupations normally requiring three years but not six years of post-comprehensive school education, with or without subordinates) and low level (normally requiring two but not three years of post-comprehensive school education with or without subordinates) and self-employed professionals such as architects and lawyers.

Cases and controls

To identify pSS cases with pre-diagnostic information in the health surveys, the MPMP and the MDCS were linked to the Malmö Sjögren's Syndrome Register (MSSR). The MSSR is a research database, in which consecutive patients with pSS have been included since 1984. At the time of the present study 409 patients were included in the cohort. Since 2002 only patients fulfilling the AECG criteria have been included. Patients included before 2002 have been assessed retrospectively and classified according to the AECG criteria. Only patients with a first pSS diagnosis after inclusion in the MPMP or MDCS, and who fulfilled the AECG criteria, were included in the present study.

The earliest available data were used for patients included in both surveys before diagnosis of pSS. Retrospective data on symptom onset obtained at the time of diagnosis were collected from the MSSR, thus were independently collected from the exposure data. Four controls for each case, matched for sex, age and time of inclusion, were obtained from the corresponding health survey. Controls were

randomly selected from those who fulfilled the matching criteria using specially designed software. This nested case-control study design, based on the same source populations, has previously been used in several studies of predictors of RA [27–30] and giant cell arteritis [31].

Statistics

Potential predictors of pSS were analyzed using conditional logistic regression (SPSS version 22). Each case and the corresponding controls were given a group number that was entered into the logistic regression models as a categorical variable. Analyses were also stratified by time to pSS diagnosis in the cases (above vs below the median). Separate analyses included only cases with documented onset of symptoms after inclusion in the health survey, and the corresponding controls.The two-sided Fisher exact test was used to analyze differences in immunologic markers after diagnosis of pSS by smoking status at inclusion in the health survey. A p value <0.05 was considered significant.

Results

Incident cases and controls

A total of 63 incident cases, patients who prior to a first diagnosis of pSS and fulfilment of the AECG criteria had been included either in the MPMP, the MDCS, or both, were identified. These are, from here, designated “pre-pSS patients”: 252 controls were obtained from the corresponding health surveys. Characteristics of the patients at diagnosis are shown in Table 1. The median time between inclusion in one of the health surveys and subsequent diagnosis was 8.2 years (IQR 2.4–14.1). Median age at inclusion in the health surveys was 51 years, and median age at diagnosis was 61 years. Demographics and information on exposures for cases and controls are listed in Table 2.

Smoking and the risk of pSS

Information on smoking status was available in 60 cases. The distribution of current, former and never smokers differed between controls and pre-pSS patients with a higher proportion of former smokers among pre-pSS patients (Table 2). In conditional logistic regression analysis, current smoking at the time of inclusion in the health surveys was associated with a significantly lower risk of later being diagnosed with pSS compared to current non-smoking (OR 0.3; 95% CI 0.1–0.6). Former smoking was associated with a higher risk of being diagnosed with pSS, both compared to never smoking (OR 4.0; 95% CI 1.8–8.8) and current smoking (OR 7.8; 95% CI 3.1–19.9) (Table 3).

Sensitivity analyses

Retrospective data from the MSSR on symptom onset were available in 49 cases. There were 28 patients who had symptom onset >1 year after inclusion in the population surveys (range 2–24, median 6.5, IQR 3.25–14.75), and information on smoking status was available in 26 of these patients. In analyses restricted to these cases and their controls, current smoking was still associated with a lower risk of later being diagnosed with pSS compared with current non-smoking, and there was a trend towards an association between former smoking and subsequent pSS diagnosis (Table 3). In order to answer the question as to whether cessation of smoking was due to symptoms of pSS, we analyzed the time window between symptom onset and cessation of smoking. Among the cases with symptom onset >1 year after inclusion in the populations

Table 1 Characteristics of incident cases of primary Sjögren’s syndrome

Characteristic	Patients ($n = 63$)
Sex (male/female) (n)	5/58
Age at diagnosis of pSS (years)	61 (54–69)
Time between inclusion and diagnosis (years)	8.2 (2.4–14.1)
Time between onset of symptoms and diagnosis (years)	4.0 (1.0–6.8)
Anti-SSA seropositive	59%
Anti-SSB seropositive	41%
ANA seropositive	73%
RF seropositive	57%
Lower lip salivary gland biopsy focus score ≥1	85%

Results are presented as numbers, median (IQR) or percentage in cases with available data. Missing data on time of symptom onset in 15 patients, Sjögren’s syndrome related antigen A, Ro (anti-SSA), Sjögren’s syndrome related antigen B, La (anti-SSB) and antinuclear antibodies (ANA) in 1 patient, rheumatoid factor (RF) in 2 patients and lower lip salivary gland biopsy focus score in 6 patients. *pSS* Primary Sjögren’s syndrome

Table 2 Demographic and exposure information in pre-primary Sjögren's syndrome cases and controls

	Cases ($n = 63$)	Controls ($n = 252$)	P value**
Female sex, n (%)	58/63 (92)	232/252 (92)	1.00
Age at inclusion in the population survey, mean (IQR), years	52.6 (49.0–56.0)	52.7 (49.0–56.0)	0.94
Current smoker, n (%[a])	10/60 (17)	92/251 (37)	0.003
Former smoker, n (%[a])	32/60 (53)	63/251 (25)	< 0.001
Never smoker, n (%[a])	18/60 (30)	96/251 (38)	0.23
White-collar worker, n (%[a])	26/54 (48)	131/236 (56)	0.33
Blue-collar worker, n (%[a])	22/54 (41)	89/236 (38)	0.68
Other socioeconomic status, n (%[a])	6/54 (11)	16/236 (7)	0.28
Low level of formal education, n (%[a])	25/58 (43)	99/243 (41)	0.73
Medium/high level of formal education, n (%[a])	33/58 (57)	144/243 (59)	0.73

[a]Percentage of individuals with available data. **Mann-Whitney U test

Table 3 Association between smoking status, socioeconomic status and level of formal education and development of primary Sjögren's syndrome

		All pSS cases ($n = 63$) and matched controls ($n = 252$) OR (95% CI)	Exposed (cases/controls)	pSS cases with documented symptom onset after inclusion ($n = 28$) and matched controls ($n = 112$) OR (95% CI)	Exposed (cases/controls)
Smoking	Not current smokers	Reference	50/159	Reference	22/74
	Current smokers	0.3 (0.1–0.6)	10/92	0.2 (0.1–0.9)	4/38
	Never smokers	Reference	18/96	Reference	10/43
	Former smokers	4.0 (1.8–8.8)	32/63	1.7 (0.6–5.6)	12/31
	Current smokers	0.5 (0.2–1.3)	10/92	0.3 (0.1–1.5)	4/38
Socioeconomic status	White-collar worker	Reference	26/131	Reference	11/53
	Blue-collar worker	1.4 (0.7–2.8)	22/89	0.8 (0.2–2.5)	9/40
	Other	2.4 (0.7–8.4)	6/16	1.0 (0.3–3.2)	4/11
Education	Medium/high	Reference	33/144	Reference	11/55
	Low	1.1 (0.6–2.1)	25/99	1.4 (0.5–4.0)	13/49

Smoking status and level of formal education were assessed at inclusion in the health surveys. Patients were subsequently diagnosed with primary Sjögren's syndrome (pSS) after a median of 8.2 years (IQR 2.4–14.1) after inclusion

surveys, there were 12 patients who were former smokers. The time period between cessation of smoking and symptom onset was >5 years in all these cases.

In the whole group of pre-pSS patients who were former smokers and for whom information was available on symptom onset (n = 23), including those with symptom onset before inclusion in the health surveys, cessation of smoking occurred >5 years before symptom onset in 20 cases, 4 years before symptom onset in 1 case and after symptom onset in 1 case.

Socioeconomic status, level of formal education and the risk of pSS

Socioeconomic status, comparing blue-collar workers and white-collar workers, did not affect the risk of being diagnosed with pSS. Furthermore, there was no association between the level of formal education and subsequent diagnosis of pSS (Table 3).

Stratified analyses

To further examine the relationship between smoking or other exposures and pSS, analyses were performed separately in cases with a time period between inclusion in one of the health surveys and diagnosis of pSS that was above or below the median (8.2 years) and in the corresponding controls. The results in these subgroups were similar to those in the whole group (Table 4).

Anti-Sjögren's syndrome related antigen A, Ro (anti-SSA) antibody positivity or presence or absence of focal sialadenitis at the time of diagnosis was not significantly different between individuals who were currently or not currently smoking at the time of inclusion in the health surveys, nor between those who had ever smoked or never smoked, or those who were currently smoking, were former smokers, or had never smoked (Table 5).

Discussion

In this study, being a current smoker was associated with a lower risk of later being diagnosed with pSS. Former smokers had a higher risk of subsequently being diagnosed with pSS, compared both to never smokers and current smokers. The same pattern is seen in ulcerative colitis [18]. In Parkinson's disease, there is also an inverse association between cigarette smoking and development of the disease [20]. Interestingly, in three previous studies specifically addressing the association between smoking and pSS, there were fewer current smokers and more former smokers among pSS patients. The first study involved 355 pSS patients in whom there was a lower frequency of focal sialadenitis in lower lip biopsies and a lower frequency of anti-SSA positivity in patients with pSS who were smokers [21]. However, as the Copenhagen criteria were used, in which the presence of anti-SSA/SSB or presence of focal sialadenitis are not mandatory, several patients not fulfilling the AECG criteria were included in that study. One interpretation of that study is that within a population of individuals with sicca symptoms, smoking is negatively associated with markers of autoimmune sialadenitis.

The second study was a cross-sectional study of 140 patients, fulfilling the AECG criteria, in which there were also more former smokers and fewer current smokers among patients with pSS [22]. Finally, in a cross-sectional study of 207 pSS patients fulfilling the AECG criteria, there were fewer current smokers, but more former smokers compared to healthy controls [23]. The characteristics of the patients in that study were similar to the characteristics after diagnosis of the pSS

Table 4 Associations stratified by time from inclusion to diagnosis of primary Sjögren's syndrome

		Time period ≤8.2 years (*n* = 32) OR (95% CI)	Exposed (cases/controls)	Time period >8.2 years (*n* = 31) OR (95% CI)	Exposed (cases/controls)
Smoking	Not current smokers	Reference	27/87	Reference	23/72
	Current smokers	0.3 (0.1–0.9)	4/40	0.3 (0.1–0.8)	6/52
	Never smokers	Reference	11/58	Reference	7/38
	Former smokers	4.9 (1.6–15.2)	16/29	3.4 (1.1–10.8)	16/34
	Current smokers	0.5 (0.1–1.8)	4/40	0.5 (0.1–2.0)	6/52
Socioeconomic status	White-collar worker	Reference	13/65	Reference	13/66
	Blue-collar worker	1.4 (0.5–3.8)	11/51	1.3 (0.5–3.7)	11/38
	Other	2.0 (0.3–14.3)	2/6	2.6 (0.5–14.4)	4/10
Education	Medium/high	Reference	18/74	Reference	15/70
	Low	1.1 (0.4–2.5)	13/53	1.1 (0.4–3.2)	12/46

Relationship between smoking, socioeconomic status and level of formal education stratified by time from inclusion in the health surveys to diagnosis of primary Sjögren's syndrome

patients in the present study, except that they had a higher frequency of positive lip biopsies (99.5% compared to 85% in the present study). In three other studies, which investigated cardiovascular risk factors in patients with pSS, there were fewer current smokers in one study [32], and in the other two studies there were fewer ever smokers [33, 34], compared to controls. In the latter two, there was no information on the numbers of current and former smokers.

Smoking is shown to cause a short-term increase in salivary flow rate (SRF) but a long-term reduced SFR [35, 36]. Cigarette smoking is also shown to cause ocular dryness when comparing chronic smokers with healthy controls [37]. A potential explanation for the smaller numbers of current smokers and for the greater numbers of former smokers in cross-sectional studies of pSS is that oral and ocular dryness and pulmonary symptoms make the patients more prone to quit smoking, both due to dryness and the irritation caused by the smoke. Interestingly, in this study, cessation of smoking in the majority of pSS patients who were former smokers did not occur in close temporal proximity to symptom onset and the impact of smoking was similar in cases with a longer and shorter time to diagnosis, supporting the possibility that smoke cessation of smoking might not be a consequence of sicca symptoms. Instead, it may influence the development or progression of the disease. Still, we cannot exclude that very early dryness, occurring before the reported onset of symptoms, could influence the cessation of smoking.

Low levels of formal education and a poor socioeconomic status have been shown to predict a number of chronic disorders [17, 26, 38, 39]. In contrast, in the present study, the level of education and socioeconomic status based on current occupation had no major impact on the risk of being diagnosed with pSS. This suggests that the observed negative association with smoking is probably not due to underlying exposures related to socioeconomic status, although we cannot exclude the possibility that other life style factors contribute to the development of pSS.

Cigarette smoke contains over four thousand different compounds [40]. Nicotine is known to interact in the immune system and nicotine receptors are present on macrophages, T cells and B cells [41–43]. Many other compounds in cigarette smoke can also affect the immune system [44]. Several studies have shown that cigarette smoke has effects on the immune system, which reasonably could affect the development of different diseases. As cigarette smoke,

Table 5 Immunologic markers at primary Sjögren's syndrome diagnosis or later by smoking status at inclusion *n* (% of available)

	Current smoker	*P* value*	Former smoker	*P* value*	Never smoker	*P* value*
Anti-SSApositive, *n* (%)	8 (80)	0.29	18 (56)	0.60	10 (56)	0.78
Anti- SSB positive, *n* (%)	7 (70)	0.07	11 (34)	0.43	6 (33)	0.57
ANA positive, *n* (%)	9 (90)	0.26	20 (63)	0.08	15 (83)	0.35
RF positive, *n* (%)	4 (44)	0.72	16 (50)	0.60	12 (67)	0.26
Autoantibody[a] negative	1 (10)	1.00	7 (22)	0.16	1 (6)	0.26
Positive lower lip biopsy, *n* (%)	8 (80)	0.63	23 (82)	0.71	15 (94)	0.41

Smoking status at inclusion in the health surveys. Primary Sjögren's syndrome was diagnosed at a median of 8.2 years (IQR 2.4–14.1) after inclusion. *Two-sided Fisher exact test. [a]Patients who were negative for anti-Sjögren's syndrome related antigen A, Ro (anti-SSA), anti-Sjögren's syndrome related antigen B, La (anti-SSB), antinuclear antibody (ANA) and rheumatoid factor (RF)

among other things, is shown to affect neutrophils, macrophages, T cells, B cells, dendritic cells, and cytokine production (reviewed in [40 – 42]), it is hard to predict exactly how cigarette smoke might affect the development of pSS [44–46]. One possible way is by inhibition of B cells: several studies have identified decreased levels of salivary IgA and decreased levels of serum IgG, and decreased levels of serum IgA and IgM and increased levels of IgE in smokers [46]. Furthermore, significant increases in IgM and IgG in subjects who stop smoking have been reported [47] and these changes seem to persist in former smokers for years after cessation of smoking [48].

pSS is characterized by B-cell activation with high serum IgG-levels and a high frequency of autoantibodies such as RF, anti-SSA and anti-SSB [1]. A reason why current smoking could be protective against pSS could be a suppressing effect of smoking on the B cells directly or by reduced production of B-cell activating factor [49, 50]. In predisposed individuals who stop smoking, taking away the inhibitory effect of cigarette smoke on the B cells could lead to activation of autoreactive B cells that might trigger the development of the disease. The association between smoking and anti-citrullinated peptide antibody (ACPA)-positive RA, on the other hand, most likely reflects other mechanisms specifically related to RA, such as increased protein citrullination leading to early development of ACPA [51].

As models of autoimmune diseases and the effect of cigarette smoking, inflammatory bowel diseases are interesting because smokers have a higher risk of Crohn's disease but a lower risk of ulcerative colitis. Studies on ulcerative colitis and Crohn's disease have identified distinct cytokine profiles, whereby Crohn's disease is characterized by a T helper (Th)1 profile [52, 53], while in ulcerative colitis a Th2 cytokine profile has been observed [54, 55], with upregulated levels of IL-33 [56]. IL-33 is a nuclear cytokine of the IL-1 family, which is constitutively expressed in epithelial barrier tissues and plays an important role in Th2 immunity [57]. Recently, increased IL-33 in the salivary glands and in serum have been linked to pSS [58]. Interestingly, in a murine model, it has also been shown that exposure of cigarette smoke upregulates IL-33 but simultaneously changes the immune response of IL-33 from Th2 to Th1 response [59]. Hypothetically, this could explain why smoking may be protective against ulcerative colitis, and potentially also against pSS.

As the patients included in the MSSR are consecutive patients at the only department of rheumatology in the area, we consider the patients in the present study to be representative of the pSS population. Strengths of the present study include the study design, with exposure measured in a standardized manner before pSS diagnosis. The availability of two population-based surveys from a well-defined catchment area and the well-characterized patients with pSS in the MSSR are other important assets of the study.

Limitations of the study are mainly related to the relatively small number of cases. As the number of patients is small there is a risk that the patients are not representative of the whole pSS cohort. Unfortunately, quantitative information on smoking history (i.e. pack-years) was not available. Furthermore, smoking status was only assessed at one time point. All patients came from the same catchment area, which can influence the external validity of the study. With a disease like pSS, there is also always uncertainty about when the disease actually starts. However, in sub-analyses including only cases with symptom onset after the collection of survey data, we calculated similar point estimates on the association between smoking history and pSS, suggesting that reversed causality does not explain our findings.

Conclusion

In conclusion, this study shows that current smoking was associated with a lower risk of later being diagnosed with pSS, whereas former smoking was associated with a higher risk. This pattern is similar to that seen in some other autoimmune diseases, e.g. ulcerative colitis and Parkinson's disease, and may reflect immunomodulatory effects of smoking and cessation of smoking. These results from the first nested case-control study of smoking and pSS are consistent with previous observations from cross-sectional studies, and suggest that smoking might be protective against pSS. However, given the insidious onset of the disease, the limited number of patients included in the present study and the fact that smoking status was only assessed at one time point, the results of this study should be confirmed in subsequent prospective studies.

Abbreviations

ACPA: Anti-citrullinated peptide antibody; AECG criteria: American European Consensus Group criteria; ANA: Antinuclear antibodies; BAFF: B-cell activating factor; CI: Confidence interval; IFN: Interferon; Ig: Immunoglobulin; IL: Interleukin; IQR: Interquartile range; MDCS: Malmö Diet and Cancer study; MPMP: Malmö Preventive Medicine Program; MSSR: Malmö Sjögren's Syndrome Register; pSS: Primary Sjögren's syndrome; RA: Rheumatoid arthritis; RF: Rheumatoid factor; SFR: Salivary flow rate; SLE: Systemic lupus erythematosus; SSA: Sjögren's syndrome related antigen A, Ro; SSB: Sjögren's syndrome related antigen B, La

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to Swedish legislation (the Personal Data Act), but a limited and fully anonymized dataset containing the individual patient data that support the main analyses is available from the corresponding author on reasonable request.

Authors' contributions

PO participated in the study design, the collection of, the statistical analysis and the interpretation of the results and drafted the manuscript. ET participated in the study design, the collection of data, the statistical analysis and the interpretation of the results and helped to revise the manuscript. CT participated in the study design, the statistical analysis and the interpretation of the results and helped to revise the manuscript. LJ participated in the interpretation of the results and helped to revise the manuscript. TM participated in the interpretation of the results and helped to revise the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All participants gave their informed consent for inclusion in the MDCS, the MPMP, and the MSSR. The current study was performed in accordance to the declaration of Helsinki and was approved by the regional ethical review board for southern Sweden (Lund, Sweden: 2007/426).

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Study II

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Multiplex cytokine analyses in patients with rheumatoid arthritis require use of agents blocking heterophilic antibody activity

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Objectives: Heterophilic antibodies, such as rheumatoid factor (RF), are known to interfere with enzyme-linked immunosorbent assays (ELISAs). Treatment of rheumatoid arthritis (RA) with tumour necrosis factor (TNF)-α blockers is well established. The aims of this study were to develop a protocol for blocking the interaction of present heterophilic antibodies and to validate this procedure by evaluating the effect on correlations of cytokine levels to clinical response in RA patients treated with adalimumab.
Method: Fourteen patients with active RA were evaluated at baseline and 3 months after starting adalimumab treatment. Cytokines were analysed with a commercial 12-plex bead ELISA. To block interference by RF, a commercial blocker (HeteroBlock) was used. To determine the optimal concentration of HeteroBlock, patient sera were analysed with different concentrations of HeteroBlock. Subsequently, baseline and follow-up sera from the 14 patients were analysed and correlated with clinical outcome.
Results: Measured cytokine levels were reduced in the majority of samples when adding the blocker. The optimal concentration of HeteroBlock was 1600 μg/mL of serum. Sera with high RF levels were more prone to produce false positive values, although some RF-negative sera also demonstrated evidence of interference. HeteroBlock did not interfere with the analysis. In RA patients treated with adalimumab, changes in interleukin (IL)-6 levels between baseline and follow-up correlated with changes in erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in sera with added HeteroBlock.
Conclusions: When analysing sera from patients with RA with multiplex bead ELISA, the assay should be evaluated for interference by heterophilic antibodies, and if present corrected with, for example, HeteroBlock.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that is associated with a number of abnormalities of the immune system, including circulating autoantibodies such as anti-citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF). RF binds the Fc portion of immunoglobulin (Ig)G and can belong to any isotype of immunoglobulin (e.g. IgM, IgA, IgG, IgD, IgE). IgM-RF (1), which is the most frequent isotype analysed in clinical practice, is present in approximately two-thirds of RA patients (2). RFs constitute a subset of heterophilic antibodies that are multispecific and can bind with weak affinity to the Fc or the Fab portion of immunoglobulins (3).

Previous studies have shown aberrant cytokine patterns in patients with RA compared to healthy controls (4–8). Cytokine profiling has previously been used for studying the prediagnostic phase (6, 7) of RA as well for prognostic purposes (9) in established RA. There have also been studies of changes in cytokine patterns in response to methotrexate (MTX) (4) and etanercept (8). However, in several of these studies, no evaluation of the interference of heterophilic antibodies has been performed (10, 11).

Analysing serum samples with bead-based enzyme-linked immunosorbent assay (bead ELISA) has the advantage that it enables analyses of several cytokines simultaneously using only a small volume of serum. It is well known that RFs can interfere with ELISA (12, 13). Previous studies using bead ELISA have shown interference by RFs and several different methods to deal with this problem have been proposed, yet without a consensus on how to solve it (14, 15).

Treatment with tumour necrosis factor (TNF) inhibitors has led to major clinical improvement in many cases of severe RA (16, 17). Adalimumab is a fully human monoclonal anti-TNF antibody that has been efficacious

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in clinical trials of MTX non-responders as well as in MTX-naïve patients (18, 19). Studies suggest that anti-inflammatory effects of TNF inhibitors may also reduce RA-associated vascular co-morbidity (20, 21), possibly due to reduced systemic endothelial activation (22).

The aims of this study were (a) to develop a protocol for blocking the interaction of present heterophilic antibodies when analysing sera from patients with RA using multiplex bead ELISA and (b) to validate this procedure by evaluating the effect on correlations of cytokine levels with clinical outcome in RA patients before and after treatment with adalimumab.

Method

These studies were approved by the regional research ethics committee in Lund, Sweden (no. 544/2004; 2004-10-28) and were performed in accordance with the Helsinki Declaration. The study of patients treated with adalimumab was also approved as a phase IV clinical trial by the Swedish Medical Products Agency, and monitored according to a standard protocol by an independent monitor. This study is registered with ClinicalTrials.gov, number NCT01270087. The results on the primary endpoint have been published (22). All participating patients gave their written informed consent.

Development of a protocol for blocking heterophilic antibody interference

To block interaction with heterophilic antibodies, HeteroBlock (Omega Biologicals, Bozeman, MT, USA), a mix of purified IgG from animal sources that has been shown to block activity of heterophilic antibodies in previous studies (14, 23, 24), was used. To determine the optimal concentration of HeteroBlock, four patients with RA and known high RF levels, two RF-positive patients with Sjögren's syndrome, and one healthy control were analysed with different concentrations of HeteroBlock (0, 160, 1600, 3200 μg/mL serum) guided by previous results in the literature (14). HeteroBlock was added to the serum and samples were incubated for 30 min with shaking at 300 rpm. To evaluate the potential interference of HeteroBlock in the assay, the control serum was analysed with the same concentrations of HeteroBlock (0, 160, 1600, 3200 μg/mL serum). Additionally, a cytokine mix (Bio-Rad, Hercules, CA, USA) was added to the control serum at two different concentrations and then analysed with three different concentrations of HeteroBlock (160, 1600, 3200 μg/mL serum). After blocking and spiking with cytokines, samples were analysed using a custom-made bead ELISA 12-plex kit [IL-1b, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-15, IL-17, interferon (IFN)-γ, TNF-α] from Bio-Rad on a Luminex 200 xMAP system (Luminex, Austin, TX, USA). The analysis was performed according to the protocol provided by the manufacturer. A standard curve with two additional dilution steps compared to standard was chosen to catch low values. All samples were analysed in duplicate, and mean values were used. Data analysis was performed using Bio-Plex Manager software version 6.1 (Bio-Rad).

Inclusion of patients for treatment with adalimumab and analysis of cytokines at baseline and after 3 months

Inclusion and exclusion criteria. Fourteen consecutive patients, seen at a single centre, who fulfilled the 1987 American College of Rheumatology (ACR) classification criteria for RA (25), and for whom treatment with adalimumab was indicated according to their rheumatologist, were included in an open-label trial. They had to have been non-responders to at least one disease-modifying anti-rheumatic drug (DMARD) and have active disease (≥ 6 swollen joints in the 28-joint index, and a CRP > 8 mg/L within the past 3 months). Details on further inclusion and exclusion criteria have been published previously (22).

Clinical evaluation

Patients were evaluated for RA disease activity at baseline and after 3 months, using standard measures: number of swollen joints, number of tender joints, RF, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Health Assessment Questionnaire Disability Index (HAQ-DI), patient's assessment of pain, and patient's global assessment of disease activity).

Handling of samples

Fasting peripheral blood samples were obtained in a standardized fashion. After centrifugation (3000 rpm, 10 min), serum was removed and stored at –80°C until analysis. The mean storage time before analysis was 7.5 years. Baseline samples were thawed twice before the present analysis for pilot studies of the assay. Follow-up samples had not been thawed previously. Before analysis, samples were thawed and centrifuged at 14 300 rpm for 20 s to reduce debris.

Analyses of samples before and after treatment with adalimumab

Serum samples from the 14 patients included in the study were analysed using the 12-plex kit mentioned earlier. Samples were analysed both with and without Heteroblock (1600 μg/mL serum). All samples were run in duplicate and mean values were used. The assay was performed according to the protocol provided by the manufacturer.

Statistical analyses

Statistical calculations were performed using SPSS for Mac, version 20 (SPSS Inc, Chicago, IL, USA). In the comparison of clinical parameters and cytokine levels at baseline with those observed after 3 months of treatment, the paired T test was used for parameters with a normal distribution, and the Wilcoxon signed rank test for skewed parameters. Spearman's test was used to assess correlations between absolute values as well as between changes over time in cytokine levels and clinical parameters.

Results

Assay performance

The working range and limit of detection according to the manufacturer are presented in Supplementary Table 1 along with the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) measured in our study. The cytokines included in the statistical analyses of correlation to clinical outcome (IL-6, IL-7, and IL-8) were all in the declared working range of the assay by the manufacturer.

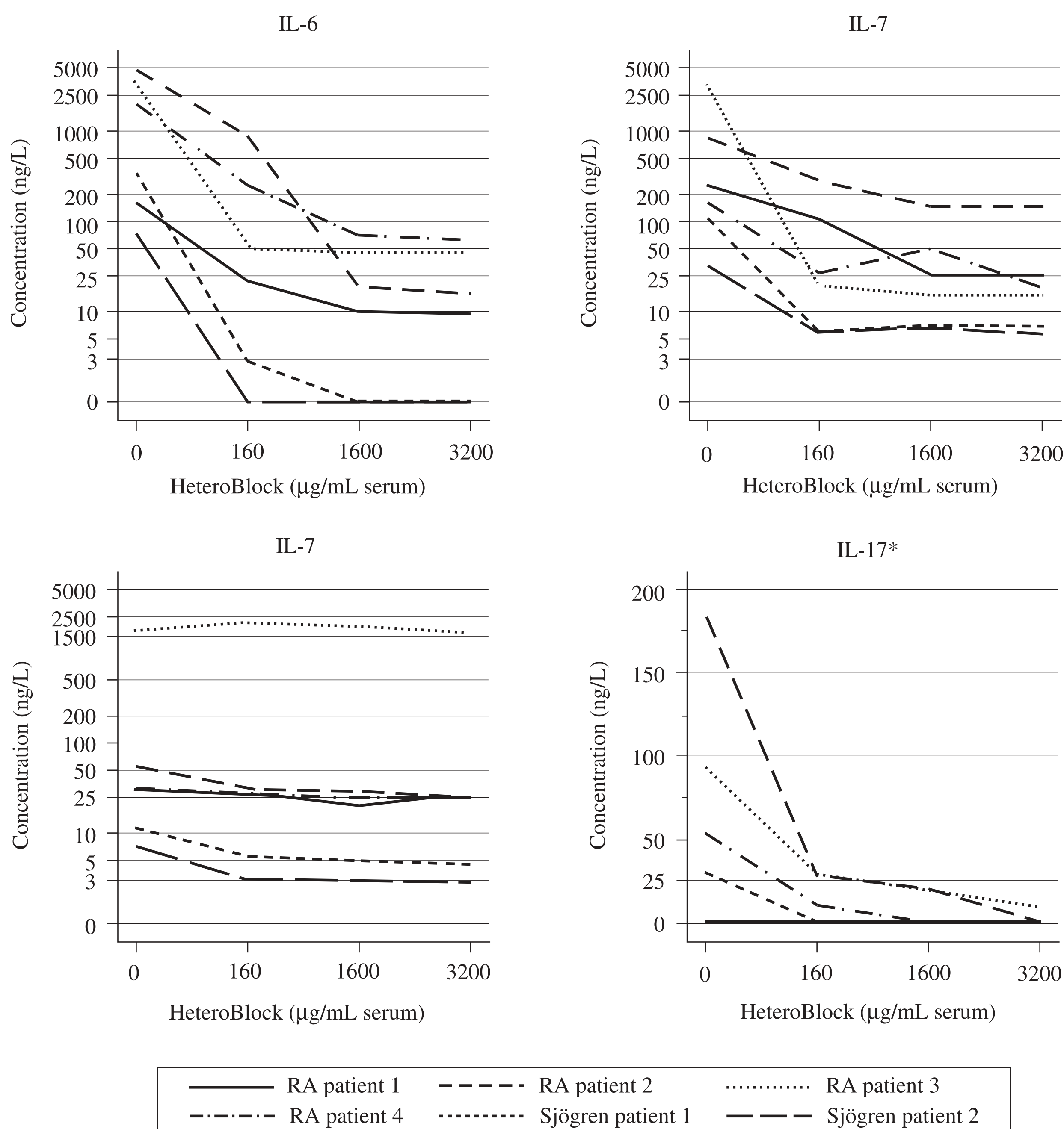

Figure 1. Measured cytokine levels in representative examples of serum from four RA patients and two Sjögren's syndrome patients with different concentrations of HeteroBlock. Logarithmic scale unless otherwise indicated. *Linear scale due to different distribution.

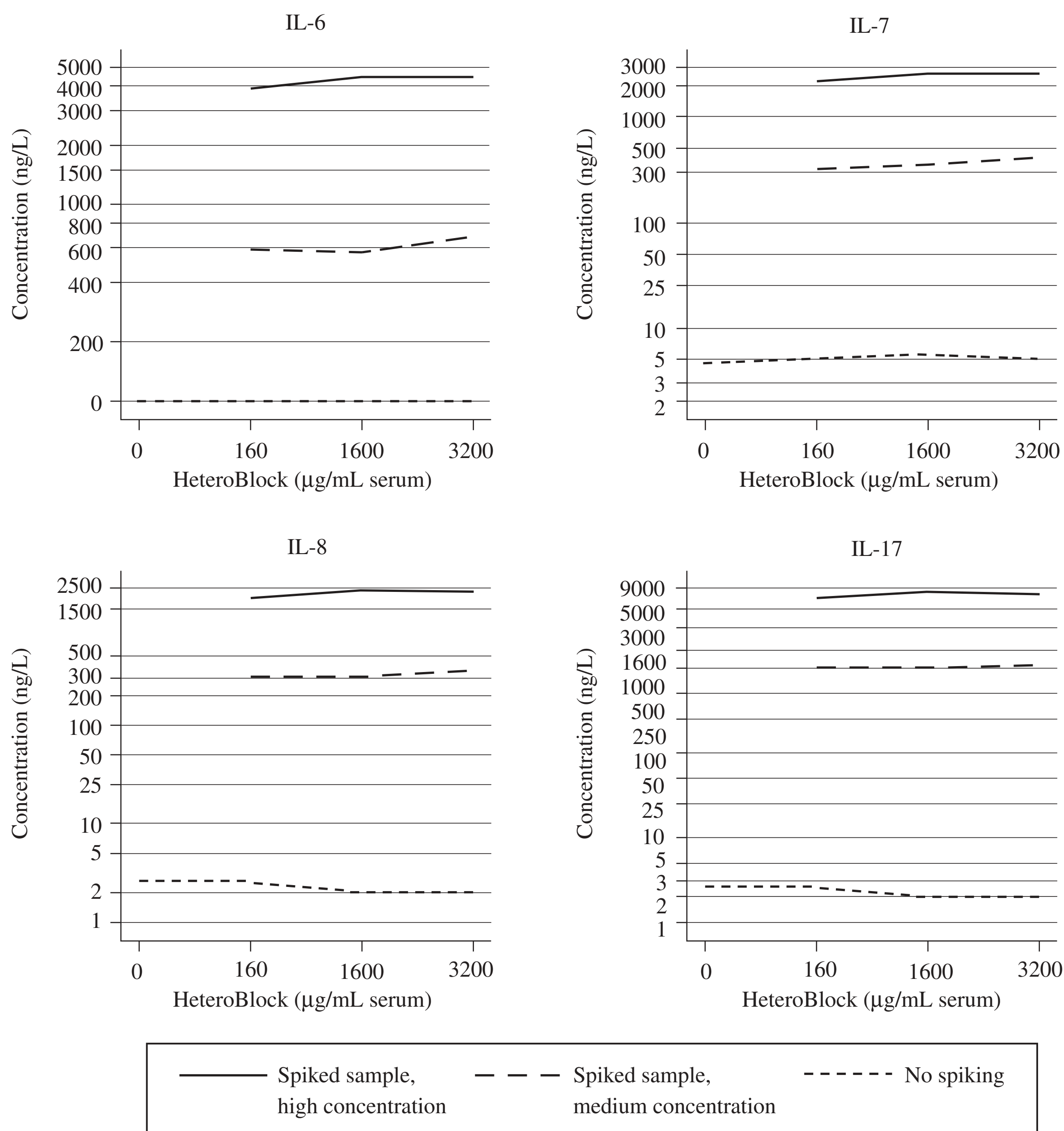

Figure 2. Control serum spiked with different concentrations of cytokines analysed with addition of different concentrations of HeteroBlock.

Recovery

Baseline samples. Recovery was between 82% and 129% when concentrations were higher than background except in one case (IFN-γ 9%). In this case the value was below the declared working range of the assay and the flourescence intensity value (FI-value) was very close to the blank (1.38 vs. 1.35); thus the result was probably due to inaccuracy of the assay in the lower spectrum (Supplementary Table 1).

Follow-up samples. In the analyses of the follow-up samples, recovery was between 69% and 139% when concentrations were higher than background (Supplementary Table 1).

Table 1. Baseline characteristics of the RA patients in the study.

n	14
Gender (female/male)	11/3
Age at inclusion (years), mean (sd)	63.7 (8.9)
Disease duration (years), median (IQR)	9.0 (2.6–11.6)
RF positive, n (%)	11 (78)
RF > 60 IU/mL, n (%)	6 (43)
RF levels (IU/mL)*, median (IQR)	84 (37–296)
Anti-CCP positive, n (%)	13 (93)
MTX treatment at inclusion, n (%)	8 (73)

RA, Rheumatoid arthritis; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; MTX, methotrexate; sd, standard deviation; IQR, interquartile range.
* In RF-positive patients.

Table 2. Clinical disease severity measures before starting adalimumab and after 3 months of treatment.

	Baseline	Follow-up at 3 months	p
DAS28	5.6 (1.3)	4.0 (1.4)	0.007
Swollen joint count (out of 28)	10.4 (5.4)	3.1 (3.7)	< 0.001
Tender joint count (out of 28)	10.0 (8.6)	5.5 (8.3)	0.07
VAS global (mm)	55.4 (24.6)	42.4 (29.3)	0.17
VAS pain (mm)	54.0 (33.1)	35.5 (30.5)	0.10
HAQ-DI	1.48 (0.73)	1.30 (0.81)	0.22
CRP (mg/L)	22 (9–39)	8 (2–22)	0.05
ESR (mm/h)	30 (18–47)	18 (9–31)	0.10

DAS28, Disease Activity Score based on 28-joint counts; VAS, visual analogue scale; HAQ-DI, Health Assessment Questionnaire Disability Index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.
Values are given as mean (standard deviation) or median (interquartile range).

Data from the protocol development part

A substantial effect of HeteroBlock on measured cytokine concentrations was seen in almost all analytes except for IL-8 (Figure 1). The effect was most obvious between unblocked samples and samples blocked with HeteroBlock 160 μg/mL of serum, but in several cases there was an additional effect when adding 1600 μg/mL. In general, no additional effect was seen when adding 3200 μg/mL of serum compared to 1600 μg/mL of serum (Figure 1). Therefore, 1600μg/mL was chosen for the subsequent investigations of sera from RA patients treated with adalimumab.

The measured cytokine levels in the control sera were stable in unspiked samples and in those spiked with different cytokine concentrations, regardless of the concentration of added HeteroBlock (Figure 2).

Data for RA patients at baseline and after 3 months with adalimumab treatment

Clinical baseline characteristics. Fourteen patients with active RA (Table 1) were started on treatment with adalimumab 40 mg subcutaneously every 2 weeks. Eight patients were on MTX at a mean dose 18.75 mg/week (range 10–25). The other six patients had previously been treated with MTX. Two of the patients had been treated with anti-TNF drugs in the past. One had stopped her only previous anti-TNF treatment just over 3 months before the start of the study. The other had received two previous anti-TNF treatments, where the last treatment was stopped more than 18 months before inclusion. Both had discontinued anti-TNF treatment due to adverse events. Four of the patients had extra-articular involvement in the form of rheumatoid nodules at inclusion, but no current vasculitis or other severe extra-articular manifestations were recorded. One patient had a history of systemic rheumatoid vasculitis.

RA clinical outcomes

The disease activity score based on 28 joint counts (DAS28) decreased from baseline to the 3-month evaluation (mean 5.6 vs 4.1; p = 0.007). A good or moderate European League Against Rheumatism (EULAR) response was seen in 8/14 patients. Disability measured by the HAQ, and inflammation, in particular when measured by CRP, were also reduced after 3 months (Table 2). Two patients achieved clinical remission according to the EULAR criteria (DAS28 < 2.6 at the follow-up at 3 months).

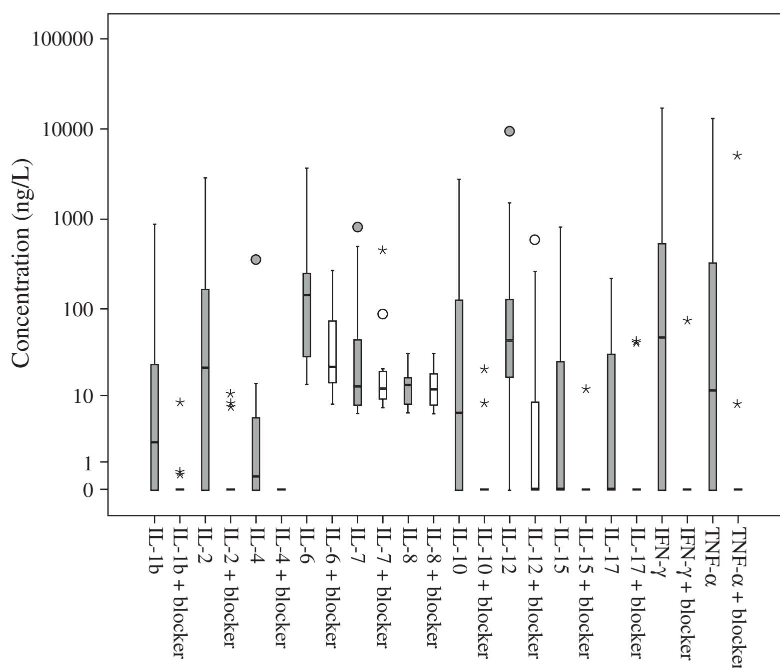

Figure 3. Patients treated with adalimumab. Baseline samples analysed with and without HeteroBlock (1600 μg/mL of serum). Stars indicate extreme outliers, circles indicate outliers, bars indicate median and interquartile range, and whiskers indicate 95th percentiles.

Table 3. Change in cytokine concentration between unblocked sera and sera blocked with HeteroBlock (1600 μg/mL serum) in patients with high vs. low RF levels.

	High RF (> 60 IU/L) n = 6			Low RF (< 60 IU/L) n = 8		
Cytokine	Median change (ng/L)	IQR	Range	Median change (ng/L)	IQR	Range
IL-1β	14.1	0.6 to 253.2	−0.5 to 865.5	0.3	0 to 6.2	0 to 30.4
IL-2	191.3	8.5 to 1028.4	0 to 2821.0	3.3	0 to 78.9	0 to 163.9
IL-4	3.1	0 to 93.5	0 to 352.9	0	0 to 2.0	0 to 14.1
IL-6	126.7	−21.1 to 1211.6	−24.0 to 3622.9	11.1	0 to 95.2	−7.3 to 126.8
IL-7	11.7	−4.3 to 378.0	−14.7 to 405.6	1.0	−1.6 to 27.2	−6.2 to 49.3
IL-8	1.2	−0.9 to 4.4	−1.0 to 7.6	0.7	0.2 to 2.5	−2.5 to 2.7
IL-10	4.1	0 to 1391.7	0 to 2724.4	17.4	0 to 110.4	0 to 264.8
IL-12 (p70)	30.9	13.3 to 3157.6	0 to 8858.6	42.8	4.1 to 118.3	0 to 387.9
IL-15	59.4	0 to 302.6	0 to 818.8	0	0 to 16.5	0 to 25.1
IL-17	0	−3.3 to 76.8	−13.0 to 179.3	0	0 to 0	0 to 46.6
IFN-γ	333.5	14.1 to 5344.6	0 to 17 403.7	12.7	0 to 210.8	0 to 1293.0
TNF-α	165.4	0 to 2328.9	0 to 7808.5	10.0	0 to 102.7	0 to 557.5

RF, rheumatoid factor.

Table 4. Measured cytokine levels (ng/L) in patients treated with adalimumab. All samples blocked with HeteroBlock 1600 μg /mL of serum.

	Baseline	3 months
IL-1b		
Median (IQR)	nd (nd–0.1)	nd (nd–0.1)
Min–max	nd–8.3	nd–9.1
IL-2		
Median (IQR)	nd (nd–1.8)	nd (nd–nd)
Min–max	nd–10.7	nd–29.5
IL-4		
Median (IQR)	nd (nd–nd)	nd (nd–nd)
Min–max	nd–nd	nd–nd
IL-6		
Median (IQR)	22.0 (14.3–91.0)	5.7 (nd–67.1)
Min–max	7.9–269.3	nd–301.0
IL-7		
Median (IQR)	12.3 (8.9–19.9)	11.7 (9.9–15.6)
Min–max	7.1–446.2	8.2–546.2
IL-8		
Median (IQR)	12.0 (7.4–19.4)	10.6 (7.6–15.7)
Min–max	5.8–31.2	6.2–33.5
IL-10		
Median (IQR)	nd (nd–nd)	nd (nd–nd)
Min–max	nd–20.1	nd–67.9
IL-12(p70)		
Median (IQR)	nd (nd–14.2)	nd (nd–0.9)
Min–max	nd–591.0	nd–1145.5
IL-15		
Median (IQR)	nd (nd–nd)	nd (nd–nd)
Min–max	nd–12.0	nd–52.6
IL-17		
Median (IQR)	nd (nd–nd)	nd (nd–nd)
Min–max	nd–43.8	nd–218.1
IFN-γ		
Median (IQR)	nd (nd–nd)	nd (nd–nd)
Min–max	nd–73.4	nd–nd
TNF-α		
Median (IQR)	nd (nd–nd)	nd (nd–nd)
Min–max	nd–5168.7	nd–8076.0

IQR, Interquartile range; nd, not detected.
Lower limits (ng/L): IL-1b = 0.11; IL-2 = 0.63; IL-4 = 0.06; IL-6 = 0.11; IL-7 = 0.11; IL-8 = 0.34; IL-10 = 0.42; IL-12(p70) = 0.55; IL-15 = 0.34; IL-17 = 1.46; IFN-γ = 1.27; TNF-α: = 1.47.

Cytokine levels before and after treatment with adalimumab

When analysing samples without a blocker, the measured cytokine levels were below the working range in 115 out of 336 cytokine results. When cytokine levels were measurable, addition of HeteroBlock reduced the measured levels of cytokines in the vast majority of samples and cytokines. The maximum difference was 17 403.7 ng/L vs. non detectable (IFN-γ). In the minority of results, where blocked values were higher than unblocked (33 out of 336 cytokine results), the difference between the results was low [maximum in absolute value 25.5 ng/L (9% of the unblocked value); maximum in relative value 350% (14.7 ng/L)]. Samples showing effects of blocking in one cytokine did so in all the others, with the exception of IL-8, if levels were measurable. The effect was most obvious in IFN-γ and TNF-α (Figure 3). Samples out of range, with values below the lowest point on the standard curve or below the blank, are presented as not detected.

IL-8 seemed to be unaffected by the added blocker. The range of values for IL-8, both blocked and unblocked, was 5.8–37.2 ng/L. Blocked samples differed from unblocked samples with a median difference of 1.2 ng/L (minimum 0.1 ng/L; maximum 14.6 ng/L) between unblocked and blocked values, in baseline and follow-up samples combined. The median percentage difference [1 – (blocked value/unblocked value)] was 12% (minimum 2%; maximum 43%).

Samples with higher IgM-RF levels (> 60 IU/mL) were more affected by adding HeteroBlock (Table 3), and samples showing extreme values unblocked all had high IgM-RF levels (> 296 IU/mL). Two patients with high IgM-RF levels showed interaction comparable with those with IgM-RF levels < 60 IU/mL. Out of three IgM-RF-negative samples, two showed a clear effect of blocking and one only a marginal effect (Supplementary Figures 1 and 2).

Table 5. Correlations between changes in serum cytokine levels over time, with and without blocking, and changes in disease activity parameters*.

	Change in CRP	Change in ESR	Change in DAS28
With blocking†			
Change in IL-6	r = 0.74 (p = 0.002)	r = 0.81 (p < 0.001)	r = 0.43 (p = 0.12)
Change in IL-7	r = 0.14 (p = 0.62)	r = 0.39 (p = 0.17)	r = –0.21 (p = 0.48)
Change in IL-8	r = 0.12 (p = 0.67)	r = 0.37 (p = 0.20)	r = 0.05 (p = 0.86)
Without blocking			
Change in IL-6	r = 0.51 (p = 0.06)	r = 0.69 (p = 0.006)	r = 0.11 (p = 0.71)
Change in IL-7	r = 0.08 (p = 0.78)	r = 0.42 (p = 0.14)	r = –0.15 (p = 0.61)
Change in IL-8	r = 0.14 (p = 0.65)	r = 0.46 (p = 0.09)	r = –0.05 (p = 0.86)

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, Disease Activity Score based on 28-joint counts.
*From baseline to 3 months after start of adalimumab.
†Heteroblock 1600 μg/mL of serum.
Spearman's rank correlation.

After blocking, only IL-6, IL-7, and IL-8 had high enough measurable concentrations to perform the statistical analysis. Serum levels of IL-6 tended to decrease from baseline to the 3-month evaluation (p = 0.24), whereas there was no major change for IL-7 and IL-8 (Table 4).

When comparing baseline and follow-up samples for the individual patients, there was a difference in the ratio between blocked and unblocked values, but the titres of IgM-RF also differed between baseline and follow-up (data not shown).

Correlation between cytokines and clinical outcome

When analysing baseline samples with no blocking agent added, there was no significant correlation between IL-6 and CRP (r = 0.19, p = 0.51). By contrast, there was a significant correlation when using blocked samples (r = 0.65, p = 0.01). The same pattern was seen when analysing follow-up samples. The decrease in IL-6, in blocked analyses, from baseline to 3 months after adalimumab initiation correlated significantly with decreases in clinically relevant laboratory markers of inflammation (CRP and ESR), whereas there was no such pattern for IL-7 or IL-8 (Table 5). In unblocked analyses, the correlation with change in IL-6 reached statistical significance for change in ESR but not for change in CRP (Table 5).

Discussion

Bead ELISAs are being increasingly used for effective simultaneous multiplex cytokine assessments in investigations of autoimmune diseases. Interference by RF has been identified as a potential problem. We present here an attempt to reduce false highly positive values due to interaction of heterophilic antibodies, in a multiplex cytokine assay. Using this method in patients with RA treated with adalimumab, we have demonstrated that measured IL-6 levels had a stronger correlation with decreases in ESR and CRP than measured IL-6 levels in unblocked samples. In addition, there was only a correlation between levels of IL-6 and CRP in the analysis with HeteroBlock. As IL-6 is known to regulate the production of CRP (26), this suggests that the biological effect of IL-6 is better reflected by the analysis including HeteroBlock. This study illustrates the importance of being aware of the risk of interference with heterophilic antibodies when analysing sera from patients with RA. In this study, HeteroBlock reduced false positive values when added in sufficient concentrations. The optimal concentration found was higher than previous studies have shown to be effective (14, 23, 24). Possible reasons for the discrepancy are that there may have been patients in this study with higher titres of heterophilic antibodies, and that the detection/capture antibody pairs in the present assay were more prone to interfere with heterophilic antibodies. Although optimal blocking was observed at 1600 μg/mL of serum, the minimal effective concentration of HeteroBlock might be in the range between 160 and 1600 μg/mL of serum, as the effects of other concentrations were not explored further in this study. Even though cytokine levels were not detectable after blocking, the unblocked values differed between baseline and follow-up samples in the same patient. One possible explanation is that the production of RF and other immunoglobulins varies over time and that the binding capacity of heterophilic antibodies is influenced by other factors (27).

HeteroBlock did not interfere with the analysis itself to any major extent, as cytokine levels in serum from the healthy controls, both spiked and unspiked, were stable despite different concentrations of HeteroBlock. IL-8 stood out in this analysis as no extreme values were found and there was no effect when adding HeteroBlock. The reason for this may be that the capture and/or detection antibodies differed from the others used in the present kit and that they were less prone to be bound by heterophilic antibodies/RF (24). The concentration of IL-8 in this study

(5.8–33.5 ng/L) was in the normal range according to the reference values (< 2–62 ng/L) in our local clinical laboratory using the Immulite® 1000 immunoassay system (Siemens Medical Solutions Diagnostics, Los Angeles, CA) and according to Bio-Rad's own reference (0.4–116 ng/L). We did not find any correlation between IL-8 and clinical baseline parameters or any significant reduction in IL-8 after treatment for 3 months, which contrasts to previous studies of patients treated with infliximab (11). Compared to the study by Klimiuk et al (11), the baseline IL-8 levels were substantially lower in our study, which also had a lower CRP threshold for inclusion. It is therefore possible that the patients studied by Klimiuk et al had a higher level of systemic inflammation and disease activity, which may explain the discrepancy, although other explanations related to methodology cannot be excluded.

When comparing the samples, the patients with the highest unblocked cytokine values were strongly positive for IgM-RF. However, not all patients who were strongly positive for IgM-RF produced high false positive values, suggesting variable affinities for RF and therefore variable interference with the assays. In addition, analyses of some samples from IgM-RF-negative (< 14 IE/mL) patients demonstrated evidence for interaction. A similar pattern has been reported previously in patients with RA (14). This is an important reminder to be cautious when analysing sera from all RA patients. Patients can be negative for IgM-RF but still have IgG-RF, IgA-RF, or IgM-RF levels under the cut-off for the RF analysis but with high avidity for certain immunoglobulins, or low avidity heterophilic antibodies that interfere in the analysis (24). The binding reactions of interacting antibodies are unpredictable and dependent on several different mechanisms (27); thus, as shown in our study, it is not necessarily the case that a serum strongly positive for IgM-RF will interact in a sandwich ELISA.

Several different ways of minimizing the effects of heterophilic antibodies have been proposed. One method is to precipitate the antibodies by adding PEG 6000 or Protein L (28). Another way is to add antibodies from mouse and other species to the sera and thus bind the heterophilic antibodies before the analysis. HeteroBlock, and several other commercial blockers such as heterophilic blocking reagent (HBR; Scantibodies Laboratories, Santee, CA, USA), immunoglobulin inhibiting reagent (IIR; Bioreclamation, Hicksville, NY, USA), and TRU Block (Amsbio, Abingdon, UK) are developments of this technique where animal (mouse) IgG is mixed with an 'active' blocking part, which is proprietary and not declared in detail. These methods have been described elsewhere in detail (15, 29). Another way of minimizing the risk of interference with heterophilic antibodies in an ELISA is to use truncated antibodies [Fab, F(ab′), or $F(ab')_2$] (30), or antibodies from species less prone to be bound by human heterophilic antibodies (24, 31).

Overall, our results are compatible with the results from Todd et al (14), except that we found a higher optimal concentration of HeteroBlock (1600 vs. 150 μg/L of serum).

Some limitations of this study are related to the small sample size. There was no analysis of cytokines with any other method as 'gold standard'. Samples had been stored for a mean of 7.5 years before the analysis, which may have affected the concentration of the cytokines (32). However, the storage time was similar for baseline samples and follow-up samples. Furthermore, the baseline samples were thawed two times more than the follow-up samples. This may also affect cytokine concentrations, as shown by de Jager et al (32). However, this effect varied for different cytokines, and in that study, IL-6 and IL-10 were stable despite several freeze–thaw cycles. This suggests that our finding of correlations between IL-6 and CRP only in the analysis including HeteroBlock would not be affected by the additional two freeze–thaw cycles of the baseline samples.

Further studies should address the reproducibility of blocking, which has been reported to be a problem by other groups (6, 33). The strengths of this study include the use of high concentrations of blocker to determine optimal blocking of interference, and the careful characterization of a relevant patient sample.

Conclusions

When analysing sera from patients with RA using multiplex bead ELISA, the assay should be evaluated systematically for its interference with RF and other heterophilic antibodies. According to this study, sera with high IgM-RF levels seem more prone to interference, but also some IgM-RF-negative sera show considerable interference, probably due to other isoforms of RF, low titre IgM-RF with high avidity, or low avidity heterophilic antibodies. We therefore conclude that all sera from all RA patients should be treated as likely to interfere in multiplex bead ELISAs. As the reported effective concentration of HeteroBlock varies enormously (3–1600 μg/mL of serum) depending on the assays used, we suggest that the optimal HeteroBlock concentration should be defined (and reported) as the first step of every bead ELISA study in each laboratory if this blocker is used. The stronger correlation between measured clinically relevant laboratory markers of inflammation and IL-6 in analyses using HeteroBlock further underlines the importance of proper methodology that takes into account the interaction with heterophilic antibodies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplementary Table S1. Assay performance
Supplementary Figure S1. Difference between measured concentrations of TNF-α in unblocked and blocked sera, by RF level.
Supplementary Figure S2. Difference between measured concentrations of IFN-γ in unblocked and blocked sera, by RF level.

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Study III

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OBSERVATIONAL RESEARCH

Smoking, disease characteristics and serum cytokine levels in patients with primary Sjögren's syndrome

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Abstract

Smoking affects several disease processes. Epidemiological studies have previously found a negative association between primary Sjögren's syndrome (pSS) and smoking. The aim of this study was to examine whether markers of disease activity and cytokine expression in pSS patients differ between ever and never smokers. Fifty-one consecutive pSS patients and 33 population controls were included in the study. Clinical and standard laboratory parameters were registered. Serum cytokines (IL-1β, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IL-33, IFN-α, IFN-γ, TNF-α, EGF, BAFF, Fas-ligand, RANTES, TGF-β1) were assessed. A positive lip biopsy was less prevalent among ever smoking patients compared to never smokers (81 vs 100%; $p=0.03$). However, except for TNF-α, which was higher in ever smokers, no differences in cytokine levels were found when comparing ever and never smoking pSS patients. Furthermore, no significant differences were found between ever and never smoking patients in the ESSDAI total score, IgG levels, or complement levels. However, IL-6, IL-12, IL-17 and IL-18 were significantly increased in pSS patients compared to controls. In this study, a negative association between ever smoking and positive lip biopsy was found, confirming previous reports. Expected differences in cytokine levels compared to controls were noted, but no major differences were found between ever and never smoking pSS patients. Taking into account the negative association between pSS diagnosis and smoking in epidemiological studies, possible explanations include a local effect of smoking on salivary glands rather than systemic effects by cigarette smoke.

Keywords Sjögren's syndrome · Cytokines · Cigarette smoking · Autoimmune diseases

Abbreviations

AECG	American-European Consensus Group
BAFF	B-cell activating factor
EGF	Epidermal growth factor
C3	Complement factor 3
C4	Complement factor 4
COPD	Chronic obstructive pulmonary disease
ESSDAI	EULAR Sjögren's Syndrome Disease Activity Index
ESSPRI	EULAR Sjögren's Syndrome Patient Reported Index
GOLD	Global Initiative for Lung Disease

Preliminary data was presented at the 14th International Symposium on Sjögren's Syndrome as a poster.

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IFN-α2a	Interferon-alpha 2a
IFN-γ	Interferon gamma
IL	Interleukin
ILD	Interstitial lung disease
IgG	Immunoglobulin G
pSS	Primary Sjögren's syndrome
RF	Rheumatoid factor
RANTES	Regulated upon activation, normal T-cell
anti-SSA	Anti-Sjögren's syndrome A
anti-SSB	Anti-Sjögren's syndrome B
TGF-β1	Transforming growth factor-beta 1
Th	T-helper cell
TNF-α	Tumour necrosis factor-α

Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterised by inflammation and destruction of the exocrine glands, classically causing oral and ocular dryness. Extraglandular organs such as lungs, kidneys, skin and nervous system may also be involved in the disease [1, 2]. The underlying pathogenesis is poorly understood, but one hypothesis states that viral triggering in combination with genetic susceptibility may result in an autoimmune attack directed against endogenous proteins and apoptotic material. The reaction results in an upregulation of proinflammatory cytokines, such as IFNα, IFNγ, and BAFF, as well as in an activation of B- and T-cells with a subsequent destruction of exocrine glands [3].

Smoking has well known negative effects, e.g. increased risk of several malignancies, development of COPD, cardiovascular disease, and rheumatoid arthritis, but on the other hand ameliorates clinical symptoms in other diseases, e.g. Behcet's disease, ulcerative colitis and Parkinson's disease [4–11]. An immunosuppressive effect by the cigarette smoke has been suggested, since not only smoke but also nicotine alone has been shown to exert effects on the immune system [12, 13].

A negative association between pSS diagnosis and smoking has previously been reported by our group and others [14–17]. It is not known whether these observations represent causality. A negative association between smoking and presence of anti-SSA antibodies and presence of focal sialadenitis in lower lip biopsies, respectively, have also been demonstrated which supports the possibility that smoking affects the disease itself [14, 16]. For example, one can argue that smoking could mask the disease by reducing the foci formation whereby patients might not fulfil the AECG or ACR/EULAR criteria, or that smoking may inhibit the development of the disease. Previous studies have shown a dose dependent effect of smoking on the presence of focal sialadenitis and have also shown that although there are no foci formations in biopsies from smoking pSS patients, there are minor inflammatory infiltrates including CD20+ cells that are not present in healthy controls, thus suggesting that smoking might inhibit the migration of lymphocytes into the salivary glands [14, 16, 18]. Cytokine aberrations have previously been demonstrated in pSS patients compared to healthy controls [19], especially in pSS patients in whom germinal centre formation has been demonstrated in salivary gland biopsies [20]. As reviewed by Arnson et al., cigarette smoke may also affect both immune cells and cytokine production [12]. To our knowledge, no other studies have been performed analysing cytokine expression in relation to smoking in pSS patients.

Taken together, the relation between pSS and smoking should be further explored. The aims of this study were (1) to investigate differences in markers of disease activity and serum cytokine levels between ever and never smoking pSS patients and (2) to assess differences in cytokine levels between pSS patients vs population-based controls.

Materials and methods

Patients and controls

At the Department of Rheumatology, Skåne University Hospital Malmö, Sweden, consecutive patients with pSS have been followed and registered since 1984. The register entailed 380 patients at the time of the study. Fifty-one consecutive outpatients who had been diagnosed with pSS by a specialist in rheumatology and fulfilled the American-European Consensus Group (AECG) criteria [21], seen from May to December in 2012, at our department were included in the study (females 49/51, median age 61, IQR: 52; 69). Clinical and laboratory parameters were assessed according to a structured protocol including ESSDAI (EULAR Sjögren's Syndrome Disease Activity Index) and ESSPRI (EULAR Sjögren's Syndrome Patient Reported Index) [22]. Median ESSDAI-total value was 7, (IQR: 1; 10). Forty of the patients had performed a lower lip biopsy of which 37 were positive. A positive lip biopsy was defined according to the AECG criteria (i.e. ≥ 1 focus of 50 cells or more per 4 mm^2). Anti-SSA positivity was found in 40/51 patients. Demographic and clinical data on patients and controls are summarised in Table 1. When investigating the effect of smoking on presence of positive lip biopsy and presence of SSA/SSB antibodies, smoking status at the time of diagnosis was used since the biopsies and analysis of SSA/SSB antibodies were performed at the time of diagnosis. The patients were also investigated for presence of chronic obstructive pulmonary disease (COPD) and interstitial lung disease (ILD) as part of another study, as previously reported [23]. Briefly, COPD

Table 1 Demographic characteristics of 51 consecutive patients and 33 controls

	Cases ($n=51$)	Controls ($n=33$)
Age, years	61 (52; 69)	47 (39; 61)
Sex, females	49/51 (96)	19/33 (58)
Current/not current smokers	4/51 (8)	7/33 (21)
Ever/never smokers	24/51 (47)	NA
Fulfilling the AECG for pSS	51/51 (100)	NA
Fulfilling the ACR/EULAR criteria for pSS	51/51 (100)	NA
Disease duration, years	12 (6; 18)	NA
Anti-SSA antibody seropositives	40/51 (78)	NA
Anti-SSB antibody seropositives	24/51 (47)	NA
ANA seropositives	40/51 (78)	NA
RF seropositives	26/51 (51)	NA
IgG, g/l	13.0 (10.1; 15.5)	NA
C3, g/l	1.01 (0.86; 1.20)	NA
C4, g/l	0.18 (0.13; 0.21)	NA
Lower lip biopsy, focus score ≥ 1%	37/40 (93)	NA
ESSPRI total score	6 (5; 7)	NA
ESSDAI total score	7 (1; 10)	NA
Nonexocrine symptoms/signs, any of the below %	25/51 (49)	NA
Lymphadenopathy and/or lymphoma ever %	3/51 (6)	NA
Arthritis ever %	4/51 (8)	NA
Cutaneous symptoms ever %	10/51 (20)	NA
Interstitial lung disease ever[a] %	9/51 (18)	NA
Chronic obstructive lung disease ever %	21/51 (41)	NA
Renal involvement ever %	4/51 (8)	NA
Myositis ever %	0/51 (0)	NA
Peripheral nervous system involvement ever %	1/51 (2)	NA
Raynaud phenomenon ever %	4/51 (8)	NA

Disease characteristics of the 51 consecutive patients with pSS. Values are presented as n/n available (%) or median (IQR) unless otherwise specified

pSS primary Sjögren's syndrome, *AECG* American-European Consensus Group criteria, *ACR* American College of Rheumatology, *EULAR* European League Against Rheumatism, *ANA* antinuclear antibody, *RF* rheumatoid factor, *IgG* immunoglobulin G, *C3* complement factor 3, *C4* complement factor 4, *ESSPRI* EULAR Sjögren Patient Reported Index, *ESSDAI* EULAR Sjögren Disease Activity Index, *EULAR* European League Against Rheumatism, *ILD* interstitial lung disease

[a]Defined as presence of peripheral traction bronchiectasis, honey combing or ground glass opacities

was defined according to the Global Initiative for Lung Disease (GOLD) criteria [24] and ILD was defined as presence of ground glass attenuation, traction bronchiectasis, or honeycombing in high-resolution CT scans.

Population-based controls, living in the city of Malmö or its surroundings were randomly selected from the Swedish population register and asked via mail if they were willing to participate in the study. If informed consent was received, the subject was invited to the Department of Rheumatology outpatient clinic, where data on age, sex, medical history, medication, present pregnancy and current smoking were registered, and a physical exam was performed. Thirty-three controls were included (females 19/33, median age 47, IQR 39; 61).

Cytokine analyses

Serum samples from patients and controls were obtained and stored at − 80 °C until analysis. Since, to our knowledge, no other studies on cytokine expression in relation to smoking and pSS previously have been published, the selection of cytokines was based on findings in previous studies analysing cytokines in pSS patients compared to healthy controls (e.g. BAFF, IFNg, IFNa, EGF) and cytokines stimulating different pathways: T-cell activating cytokines (e.g. RANTES, IL-2), B-cell activating cytokines (e.g. BAFF, IL-4, IL-10, IL-6), the Th-17 pathway (IL-17), and general proinflammatory cytokines (e.g. IL-1B, TNF-α, IL-6, IFNg).

The serum samples were analysed with four different panels, all using the Meso-Scale platform. Panel 4 was purchased from Meso-Scale for Interferon-α2a (IFN-α2a Ultra-sensitive kit, K151ACC). Panel 1–3 were in-house made assays. Panel 1 included B-cell activating factor (BAFF), Epidermal Growth Factor (EGF), Fas-ligand, Interleukin-3 (IL-3), IL-33, Regulated upon Activation, Normal T-cell Expressed and presumably Secreted (RANTES), and Transforming growth factor β 1 (TGF-β1). Panel 2 included IFN-γ, IL-2, -6, -8, -10, -12, -17, -18, -1β, tumour necrosis factor-α (TNF-α). Panel 3 was IL-4. Further details on these assays are given in the Supplementary text. Concentrations were calculated with Discovery Workbench software (Meso-Scale) from calibration curves using four-parameter logistic fit.

Based on previous reports, the panels were assessed for proneness to interaction with heterophilic antibodies using pooled IgM/IgA rheumatoid factor (RF) positive sera and pooled healthy control sera with and without HBR Plus (Scantibodies Laboratory, Santee, CA, USA) without any significant difference in cytokine levels [25, 26]. Subsequent analyses were performed without any additional blocker.

Statistics

When analysing differences in cytokine levels between ever and never smokers as well as between cases and controls the Mann–Whitney *U* test was used. Spearman's rank test was used for correlations. Chi^2 test was used for comparison of binary parameters. A *p* value below 0.05 was considered significant for all analyses. Separate analyses included cases with shorter disease duration (above median) and anti-SSA seropositive patients.

The statistic calculations were performed using SPSS version 22 for Macintosh.

Results

Amongst the 51 patients, 47% were ever smokers (8% current smokers, 39% former smokers) (Table 1). Amongst ever smokers at the time of diagnosis, significantly fewer patients had a focal sialadenitis (81 vs 100%; $p = 0.03$) (Table 2). The ESSDAI total score, the ESSPRI total score, IgG, C3, and C4 levels did not significantly differ between ever and never smoking pSS patients (Table 2). Levels of IL-6, IL-12, IL-17 and IL-18 were significantly increased in pSS patients compared to controls whilst no major differences between pSS patients and controls for the other cytokines were found (Table 3).

When comparing ever and never smoking pSS patients, only TNF-α levels were significantly higher in the former group (Table 4). Also, when analysing only anti-SSA positives as well as pSS patients with shorter than median disease duration amongst pSS patients, a similar lack of association was found. No significant difference was observed in cytokine levels between patients with or without COPD, or ILD, respectively.

Thirty-three controls (median age 47 (range 39–61 years), 19 females) were included, of whom 21% were current smokers (Table 1). Amongst controls, sex and age correlated poorly to cytokine levels. In patients with pSS, disease duration was negatively correlated to IL-10 ($r = -0.32$, $p = 0.02$), IL-12 ($r = -0.34$, $p = 0.02$) and TNF-α ($r = -0.40$, $p = 0.004$) and positively to TGF-β1 ($r = 0.29$, $p = 0.04$). There were no significant correlations between the ESSDAI total score and serum cytokine levels (data not shown). Current smokers entailed only four patients, therefore statistical analyses were not performed on this group separately.

Table 2 Comparison of clinical parameters, IgG levels and complement levels between never smoking and ever smoking pSS patients

	Ever smokers (pSS) $n = 24$	Never smokers (pSS) $n = 27$	*p* value^{+}
Focal sialadenitis	14/17 (82)	23/23 (100)	0.03*
Anti-SSA- and or SSB-positive	20/24 (83)	20/27 (74)	0.43
ESSDAI	7.5 (1.5; 10)	7 (1; 11)	0.85
ESSPRI	6 (5; 7)	6 (4; 8)	0.68
IgG (g/l)	12.9 (10.1; 17.2)	13.0 (10.1; 15.2)	0.62
C3 (g/l)	1.02 (0.92; 1.22)	0.99 (0.84; 1.16)	0.60
C4 (g/l)	0.19 (0.13; 0.21)	0.16 (0.13; 0.20)	0.40

Values are presented as median (IQR) or n/n available (%)

*$p < 0.05$

$^{+}$Mann–Whitney *U* test

Table 3 Comparison of cytokine levels between pSS patients and controls

	Cases pg/ml, median (IQR) $n = 51$	Controls, pg/ml, median (IQR) $n = 33$	p value^{+}
IL-1β	0 (0; 0)	0 (0; 0)	0.92
IL-2	0 (0; 25.0)	0 (0; 24.4)	0.86
IL-3	0 (0; 67.8)	0 (0; 88.7)	0.84
IL-4	0 (0; 0)	0 (0; 0)	0.20
IL-6	25.2 (14.0; 30.9)	15.3 (10.6; 22.0)	0.003**
IL-8	19.9 (15.9; 22.8)	16.7 (14.1; 20.8)	0.06
IL-10	0 (0; 0)	0 (0; 0)	0.18
IL-12	7.4 (0; 10.8)	0 (0; 8.3)	0.02*
IL-17	0 (0; 51.2)	0 (0; 0)	0.002**
IL-18	294 (187.7; 500.3)	214.5 (119.0; 297.5)	0.008**
IL-33	11.2 (0; 16.1)	12.5 (0; 17.7)	0.62
IFN-α	0 (0; 0)	0 (0; 0)	0.53
IFN- γ	0 (0; 1.3)	0 (0; 1.0)	0.43
TNF-α	12.1 (5.7; 16.9)	8.5 (5.9; 11.5)	0.14
BAFF	265.6 (182.4; 376.2)	276.1 (142.9; 391.6)	0.79
EGF	139.8 (60.0; 227.1)	136.4 (93.2; 177.3)	0.89
Fas ligand	9.5 (7.2; 15.3)	11.4 (6.9; 15.2)	0.48
RANTES	15673.7 (11374.1; 25397.6)	17073.0 (13345.9; 20037.8)	0.56
TGF-β1	22.4 (6.7; 35.6)	12.7 (9.2; 36.6)	0.75

*$p < 0.05$

**$p < 0.01$

$^{+}$Mann–Whitney U test

Table 4 Comparison of cytokine levels between never smoking and ever smoking pSS patients

	Ever smokers (pSS) $n = 24$ pg/ml, median (IQR)	Never smokers (pSS) $n = 27$ pg/ml, median (IQR)	p value^{+}
IL-1β	0 (0; 0)	0 (0; 0)	0.30
IL-2	0 (0; 28.4)	0 (0; 19.2)	0.85
IL-3	0 (0; 0)	0 (0; 96.9)	0.36
IL-4	0 (0; 0)	0 (0; 0)	1.0
IL-6	25.0 (14.4; 29.4)	25.2 (13.0; 36.7)	0.94
IL-8	21.8 (15.9; 24.1)	18.3 (15.5; 21.4)	0.18
IL-10	0 (0; 0)	0 (0; 39.3)	0.74
IL-12	9.7 (5.6; 12.9)	7.1 (0; 9.8)	0.20
IL-17	0 (0; 75.7)	39.0 (0; 46.6)	0.86
IL-18	364.3 (250.4; 659.3)	234.5 (166.3; 500.3)	0.06
IL-33	11.2 (0; 19.3)	11.2 (0; 15.6)	0.95
IFN-α	0 (0; 0)	0 (0; 0)	0.51
IFN-γ	0.7 (0; 1.3)	0 (0; 1.0)	0.58
TNF-α	13.8 (7.2; 20.8)	7.5 (4.9; 15.9)	0.03*
BAFF	256.1 (191.9; 375.2)	265.6 (156.7; 388.3)	0.95
EGF	154.8 (55.4; 252.0)	133.9 (70.8; 202.8)	0.53
FAS ligand	10.3 (7.3; 16.6)	9.2 (6.5; 12.6)	0.50
RANTES	17051.1 (11883.2; 27559.6)	15443.0 (9730.2; 19827.9)	0.46
TGF-β1	21.2 (4.2; 26.9)	22.4 (9.3; 55.0)	0.14

*$p < 0.05$

$^{+}$Mann–Whitney U test

Discussion

In this study, a negative association between a history of smoking and focal sialadenitis in patients with pSS was found which is in line with previous reports. The negative association between pSS diagnosis and smoking could be due to the dryness of the oral cavity and eyes potentially causing more irritation by the smoke. Cigarette smoking is also reported to cause reduced salivary rates and alteration of the saliva composition [27, 28]. To the best of our knowledge, there are no studies investigating the effect of smoking on salivary gland biopsies in healthy controls. The reported effect on salivary flow does not reach the levels required for diagnosing pSS but might potentially decrease the already diminished salivary flow in pSS patients, thereby explaining the lower frequency of current smokers amongst pSS patients. Still, it does not explain the lower frequency of focal sialadenitis in ever smoking patients. Since the salivary glands are in close proximity to the inhaled smoke, a possible explanation for this finding could be that cigarette smoke interferes with the local immune response either by nicotine binding to nicotine receptors on immune cells or by other compounds in the inhaled smoke acting anti-inflammatory [12, 13, 29, 30].

Apart from the lower frequency of positive lip biopsy, there were no significant differences in other standard clinical and laboratory characteristics between ever and never smokers. Furthermore, there were no major differences in cytokine levels, except for TNF-α, which was higher in ever smokers. The latter finding should be interpreted with caution. The TNF-α levels were generally low, and if smoking was indeed associated with a higher degree of systemic inflammation, one would expect other proinflammatory cytokines to be significantly increased as well in ever smokers. Also, given the numerous statistical analyses, this single statistical significance should not be over-interpreted.

An increase in several proinflammatory cytokines (IL-6, IL-12, IL-17, IL-18) was observed in pSS patients compared to controls. pSS is a disease characterised by an insidious onset and slow progression of exocrinopathy. Since the exocrine inflammation is mainly local, most previous studies have measured cytokine expression in biopsies [31, 32] and saliva [33] or production by peripheral mononuclear cells [34]. However, several other studies have also shown cytokine aberrations in serum including the increase in IL-6, IL-17, and IL-18 in the current study [35–39]. Furthermore, regarding the observed increase in IL-12 in pSS patients, polymorphisms of the IL12A gene have been shown to be associated with pSS in a genome-wide association-study [40].

The observed difference in cytokine expression between pSS patients and controls in our study is thus an expected finding. However, the lack of difference in BAFF levels between patients and controls was unexpected since BAFF is considered a hallmark of pSS in several studies [41, 42] and considered as a potential biomarker [43]. A possible explanation might be that these consecutive patients had a lower disease activity than patients in previous studies or that the in-house made kit was not specific enough. Problems with analysing BAFF due to lack of specificity for BAFF caused by posttranslational glycosylations or alternative spliced forms have previously been reported [44].

The type I interferon system is activated in pSS and is thought to play an important role in the disease development [45, 46]. Type I interferons consists of at least 17 different subtypes, of which there are 13 different subtypes of IFNα. In this study, IFNα2a was investigated. Despite choosing an ultra-sensitive kit, the majority of samples were below the measurable range. This is a common problem and a reason why mRNA from interferon-sensitive genes, the so-called IFN-signature, is often measured rather than IFNα itself. Unfortunately, mRNA was not available in this study. Analysing the IFN-signature in salivary gland cells or monocytes from pSS patients with different smoking exposures would be interesting, since smoking has been shown to suppress the effect of type I IFNs [47].

Most patients included in the study had a longstanding disease (median disease duration 12 years) and disease duration correlated negatively to the proinflammatory cytokines IL-10, IL-12 and TNF-α and positively to the anti-inflammatory cytokine TGF-ß, indicating that the disease develops towards a less inflammatory state over time.

In line with previous reports [14, 16], we found a lower frequency of positive lip biopsy among ever smokers at the time of diagnosis. However, we did not find evidence that ever smoking affects cytokine expression, IgG levels, complement levels or disease activity, measured by the ESSDAI-score, in pSS patients. It is possible that cytokine concentrations and ESSDAI-scores would have differed between ever and never smokers as well if measured at time of diagnosis. Another possible explanation could be that smoking might affect inflammation locally in the salivary glands rather than the systemic inflammation of the disease. Also, the group of currently smoking patients was small in this study, which makes it difficult to draw conclusions about temporary effects of current smoking on cytokine levels. Since current smokers are underrepresented in epidemiological data, it would be of interest in future studies to compare a larger group of currently smoking pSS patients with former and never smoking patients concerning cytokine levels.

The study has some limitations, including the limited sample size of the study and the low number of current smokers amongst the pSS patients. Furthermore, the majority of the patients had long-standing disease, and we cannot exclude that cytokine patterns, and their relation to smoking

history, may be different in recently diagnosed patients. The cross-sectional study design is also a limitation. Finally, the control group was not exactly matched on sex and age. Strengths of the study include the use of consecutive patients in standard follow-up, likely representative of the general pSS population and the validation of the assays concerning possible interference by rheumatoid factor.

In conclusion, there was a lower prevalence of positive lip biopsy among pSS patients with a history of ever smoking which is in accordance with previous studies. No differences in serum cytokine levels between ever and never smoking pSS patients was found. Furthermore, we found increased levels of proinflammatory cytokines (IL-6, IL-12, IL-17, IL-18) in pSS patients compared to controls as well as a negative correlation between disease duration and proinflammatory cytokines indicating that the disease develops to a less inflammatory state over time. In pSS, a local effect of smoking on salivary glands rather than systemic effects of cigarette smoke may explain the previously observed negative association between smoking and pSS.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical statement The study was approved by the Regional Ethical Review Board for Southern Sweden (Lund, Sweden; LU 2012/98). All patients gave written informed consent according to the Declaration of Helsinki.

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Study IV

Associations between cigarette smoking and disease burden, symptoms and type I interferon expression in primary Sjögren's syndrome.

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Abstract

Introduction: Several studies have shown a negative association between smoking and primary Sjögren’s syndrome (pSS), and smoking may interfere with the immune response. The purpose of this study was to investigate if smoking affects disease activity and disease phenotype in pSS.

Methods: In this cross-sectional study, consecutive pSS patients filled out the EULAR Sjögren’s Syndrome Patient Reported Index (ESSPRI) form and a structured questionnaire regarding smoking habits. EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI) scores were calculated and blood samples were analysed for type I interferon signature.

Results: Of 90 patients (93% women, median age 66.5 years), 72% were type I IFN signature positive and 6%, 42% and 53% were current, former and never smokers, respectively. No significant differences by smoking status were found regarding ESSDAI total score, activity in the ESSDAI domains or type I IFN signature. Patients with a higher cumulative cigarette consumption (>=median) had higher scores in ESSPRI total (5.0 (3.0-6.3) vs 8.0 (6.0-8.3); p <0.01) and ESSPRI sicca and pain domains. Comparing type I IFN signature negative and positive patients, the latter had significantly lower scores in ESSDAI articular domain (7/25 vs 3/64; p<0.01), ESSPRI total (7.7 (5.2-8.2) vs 6.0 (4.0-7.7); p=0.04), ESSPRI sicca domain and ESSPRI pain domain and were more often positive for autoantibodies.

Conclusion: Smoking was not associated with disease phenotype although patients with a higher cumulative cigarette consumption had worse symptoms in some disease domains. Current smokers were few making it difficult to draw any firm conclusions about associations to current smoking.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease that predominantly affects exocrine glands [1]. Affection of other organs, often termed extraglandular manifestations (EGM), is seen in approximately one third of patients [2]. Apart from female sex, the predisposing factors leading to development of pSS are not well known although genetic studies have shown associations to HLA class II genes and interferon related genes [3]. Furthermore, viral infections and sex hormones have been suggested to contribute to disease development [4]. The pathogenic process in pSS includes dysregulation of both innate and adaptive immunity. Both cell-mediated and humoral immunity is involved, the latter mirrored by hypergammaglobulinemia and production of anti-SSA and anti-SSB antibodies [4]. It is well established that type I interferons (IFNs) are important

cytokines in the pathogenesis of pSS. The presence of an activated type I IFN system is commonly evaluated by analysing the expression of a selected set of type I IFN regulated genes. The presence of an elevated expression of these genes is called an "IFN-signature". Systemic type I IFN signature is found in a large fraction of pSS patients [5]. The type I IFN signature has also been shown to be associated with presence of anti-SSA and anti-SSB antibodies, and hypergammaglobulinemia[5]. Disease activity as evaluated by the European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index (ESSDAI) has been associated to type I IFN signature previously [6,7] although some studies fail to confirm the association [5]. On the contrary, presence of the IFN signature has been associated with lower patient reported symptoms as evaluated by the EULAR Sjögren's syndrome patient reported index (ESSPRI) [5]. We recently published an epidemiological study showing that smoking was associated with a lower risk of later being diagnosed with pSS [8]. Similar findings have also been reported by others reporting a lower prevalence of smokers amongst pSS patients [9-11], and lower frequency of focal sialoadenitis [11,9] and seropositivity for anti-SSA antibodies [9,11] amongst smoking pSS patients. However, it is unclear whether the observed association is due to a local effect of smoking on the inflammation in the salivary glands or if smoking has an impact on systemic inflammation and consequently the phenotype of pSS.

Smoking has profound negative effects on health and influences the development of cardiovascular disease, chronic obstructive lung disease (COPD), oncogenesis as well as several chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease [12-15]. On the other hand, smoking seems to ameliorate certain diseases such as ulcerative colitis, Behcet's disease and Parkinson's disease [16-18]. Smoking has several known effects on the immune system which could be important for modulation of chronic inflammatory diseases [19]. To the best of our knowledge, no other study has previously investigated the relation between cigarette smoking, disease phenotype and type I IFN signature in patients with pSS.

In the present study, we wanted to a) investigate the relation between cigarette smoking and disease activity (evaluated using the ESSDAI) and burden of symptoms (evaluated using the ESSPRI) and b) investigate the relation between smoking and type I IFN signature in pSS patients.

Methods

Patients

Between September 2017 and May 2018, consecutive patients with an established diagnosis of pSS, who attended the out-patient clinic at the Department of Rheumatology, Skane University Hospital, Malmö, Sweden, were asked to participate in the study. Only patients above 18 years of age and fulfilling the American-European Consensus Group (AECG) criteria were included [20]. Exclusion criteria were concomitant fulfilment of other rheumatological diseases and inability of filling out the requested questionnaires. Patients underwent a physical examination and blood samples were drawn. Based on structured clinical evaluation and laboratory analyses the ESSDAI was calculated. In addition, patients filled out the ESSPRI form, and a structured questionnaire regarding smoking habits. Patient characteristics are summarised in table 1.

Questionnaire on smoking habits

The questionnaire on smoking habits included questions on whether the patient ever had been smoking regularly and, if the patient declared he/she had been smoking regularly, if he/she had quit smoking. The patient was considered a current smoker if answering "Yes" to the question "Have you ever been smoking on a regular basis?" and "No" to the question "Have you quit smoking?". The patient was considered a former smoker if answering "Yes" to the question "Have you ever been smoking on a regular basis?" and "Yes" to the question "Have you quit smoking?". Never smokers were defined as answering "No" to the question "Have you ever been smoking on a regular basis?" In case a patient declared ever being smoking on a regular basis, the patient was asked what year he/she started and stopped smoking as well a question on current tobacco consumption and mean tobacco consumption during years of smoking. A translated version of the form is found in the supplementary materials (Supplementary material).

Patients were divided into following groups according to smoking status: i) Current smokers ii) former smokers and iii) never smokers. Cigarette consumption, defined as pack-years was also calculated from the questionnaire. A pack-year is defined as twenty cigarettes smoked every day for one year, i.e. a cumulative consumption of 7300 cigarettes. The average number of pack-years amongst ever smokers (current+former smokers) was calculated and patients were divided into those having a cigarette consumption above or equal to the median and those with a consumption below the median.

ESSDAI and ESSPRI

The ESSDAI index and the ESSPRI index are validated indices, widely used in pSS studies, and are described in detail elsewhere. In short, ESSDAI measures disease activity in twelve different domains where different organ systems are assessed as well as laboratory analyses mirroring disease activity. The total score is calculated by adding the individual domains scores which gives a total score ranging between 0-123. Low, moderate and high disease activity is defined as a score of 0-4, 5-13 and ≥14 respectively. In the ESSPRI, patients score their symptoms in three different domains: dryness, pain, and fatigue on a Likert scale between 0-10. The total ESSPRI score is calculated as the mean of the different domains and thus has a range of 0-10 [21-23].

Laboratory analyses

Blood samples were collected during routine follow-up appointments at office hours using a standardised (non-fasting) procedure. Immunoglobulin (Ig)G, C3 and C4 were measured by nephelometry using an Immage800 (Beckham Coulter Inc., Brea, CA, USA). Data on the presence of rheumatoid factor (RF), antinuclear antibodies (ANA), anti-SSA and anti-SSB antibodies, all measured by validated methods in clinical care, is registered continuously in all pSS patients in our registry (Malmö Sjögren's Syndrome Registry) and were not re-analysed for the current study. Currently RF is measured by Phadia ImmunoCap250, anti-SSA/SSB by EuroblotOne, and ANA is by indirect immunoflourescence using HEp 2010 cells as substrate.

RT-PCR analysis of type I IFN signature

Blood was collected in clotting tubes for serum preparation and in PAXgene RNA tubes (PreAnalytix, Hombrechtikon, Switzerland) for whole blood RNA analysis. Samples were stored in -80°C until analysis. RNA isolation, cDNA preparation and RT-PCR were performed according to the manufacturer's protocol. The protocol and selection of expressed genes is previously described in detail [5]. The type I IFN induced genes analysed include IFI44, IFI44L, IFIT1, IFIT3 and MxA. Patients were divided into groups that were positive or negative for type I IFN using a threshold of mean healthy controls (HC) + 2 $S.D_{HC}$ based on the previous analysis [5].

Statistics

Due to small sample sizes, continuous data was generally considered non-normally distributed and thus data is presented as median (q1-q3) and the Kruskal-Wallis test was used for multiple group comparisons and the Mann Whitney U-test for comparison between groups. For continuous data including more than 80 cases, data were analysed for normality by visual inspection of histograms and Q-Q-plots and Shapiro-Wilk test. If normally distributed, data is presented as mean (+/- standard deviation (SD)) and the one-way analysis of variation (ANOVA) was used for multiple group comparisons and the independent samples T-test for comparison between groups. The Chi^2-test or Fisher's exact test were used for discrete variables. A p-value <0.05 was considered significant. The statistical analyses were performed using SPSS version 22 for Mac.

Results

Of 109 consecutive patients, 90 agreed to participate and were included in the study. Median age was 66.5 years (q1-q3: 51.8-73.0), 93% were females. In addition to fulfilling the AECG criteria, all patients also fulfilled the American College of Rheumatology (ACR)/EULAR criteria for pSS [24].

Smoking habits and IFN signatures of the study population

No patient declared any tobacco smoking apart from cigarette smoking. Six percent of pSS patients were current smokers, whilst 41% and 53% were former and never smokers, respectively. The median time since smoke cessation amongst former smokers was 27 years (q1-q3: 16-39). The median cigarette consumption amongst ever smokers (former + current smokers) was 8.8 pack-years (q1-q3: 4.0-19.0). 72% of pSS patients showed a type I IFN signature. Further characteristics are found in table 1.

Table 1:
pSS patient characteristics.

	n =	
Females (%)		84/90 (93)
Age (years)	90	66.5 (51.8-73.0)
Current smoker (%)		5/90 (6)
Smoke duration, years	5	48 (36.5-59.5)
Pack-years, years	5	25 (11.3-40.2)
Former smokers (%)		37/90 (41)
Smoke duration, years	37	17 (11.0-30.0)
Pack-years, years	37	8.1 (3.4-17.8)
Time since smoking cessation, years	37	27 (16-39)
Time between smoke cessation and diagnosis, years	37	13 (6.5-21)
Time between smoke cessation and sicca symptoms, years	17	14 (1-21)
Never smoker (%)		48/90 (53)
Ever smokers (current+former smokers) (%)		42/90 (47)
Pack-years, years	42	8.8 (4.0-19.0)
Smoke duration, years	42	19.5 (11.8-35.0)
Years diagnosed with pSS, years	90	13 (5.0-21.3)
Years since first sicca symptom, years	41	15 (5.0-26.0)
Anti-SSA seropositives (%)		78/90 (87)
Anti-SSB seropositives (%)		53/90 (59)
ANA seropositives (%)		70/88 (80)
RF seropositives (%)		62/88 (71)
Focal sialoadenitis (%)		62/68 (91)
IgG, g/L	89	14.8 (+/- 5.35)
C3, g/L	89	0.94 (+/- 0.21)
C4, g/l	89	0.18 (+/- 0.06)
ESSDAI total	89	3 (1.0-7.5)
ESSDAI high activity		7/89 (8)
ESSDAI moderate activity		30/89 (34)
ESSDAI low activity		52/89 (58)
ESSPRI total	89	6.7 (4.7-8.0)
Type I IFN positive		64/89 (72)

Data are presented as *n* cases/*n* total (%). For continuous data: mean (+/- SD)/median (q1-q3). ANA: Anti-nuclear antibodies, C: complement component, CNS: central nervous system, ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index, ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index, IFN: interferon, IgG: Immunoglobulin G, PNS: peripheral nervous system, RF: rheumatoid factor.

Smoking habits, ESSDAI/ESSPRI and patient characteristics

No significant differences in ESSDAI total score or activity in the ESSDAI domains were found between never, former or current smokers, nor between patients with a cigarette consumption as evaluated by pack-years below vs. ≥ 8.8 pack-years (Table 2).

When comparing ESSPRI sicca domain scores of never (7.0; q1-q3: 5.0-8.0), former (8.0; q1-q3: 5.0-9.0) and current smokers (9.0; q1-q3: 8.5-9.5) there was a statistically non-significant trend (p=0.06) towards higher values amongst current smokers. Comparing patients with a low (<8.8 pack-years) and high (≥ 8.8 pack-

years) cigarette consumption, a significantly higher ESSPRI total score (5.0 (q1-q3 3.0-6.3) vs 8.0 (q1-q3 6.0-8.3) p=0.01), ESSPRI sicca score (vs 6.0 (q1-q3: 5.0-9.0) vs 8.0 (q1-q3: 8.0-9.0) p=0.046) and ESSPRI pain score 4.0 (q1-q3: 2.0-5.0) vs (7.0 (q1-q3: 5.0-9.0) p=0.01) were found in the high consumption group (Table 2). A statistically non-significant trend towards a lower frequency of focal sialadenitis amongst current smokers (3/5 (60%)) compared to former smokers (22/24 (92%)) and never smokers (36/38 (95%)) was found (p=0.06) (Table 2). Amongst former smokers, there were no significant differences in total ESSDAI score, presence of activity in the ESSDAI domains or total ESSPRI score comparing patients below median (27 years) and ≥ median time since smoke cessation (data not shown).

No statistically significant differences in type I IFN signature positivity and smoking status were found although there were numerically fewer type I IFN positive patients amongst current smokers (40%) compared to former (78%) and never (70%) smokers (p=0.16) (Table 2). Additionally, no statistically significant differences were found in levels of IgG, C3, C4, presence of anti-SSA, anti-SSB, ANA, or RF by cigarette consumption (Table 2).

Table 2.
Associations between smoking habits and clinical characteristics in pSS patients.

	Never smokers, n=48	Former smokers, n=37	Current smokers n=5	p-value	Pack-years < 8.8 years, n=19	Pack-years ≥ 8.8 years, n=23	p-value
ESSDAI total	3 (1.0-8.0)	4 (1.0-7.5)	1.0 (0.0-8.5)	0.55	4 (1.0-7.0)	4.0 (0.0-9.0)	0.87
ESSDAI activity in each domain, *n* (%)							
Constitutional	6/47 (13)	7/37 (19)	0/5 (0)	0.61	3/19 (16)	4/23 (17)	1.0
Lymphadenopathy	3/47 (6)	1/37 (3)	0/5 (0)	0.71	1/19 (5)	0/23 (0)	0.45
Glandular	5/47 (11)	5/37 (14)	0/5 (0)	0.86	1/19 (5)	4/23 (17)	0.36
Articular	4/47 (9)	4/37 (11)	2/5 (40)	0.12	4/19 (21)	2/23 (9)	0.38
Cutaneous	3/47 (6)	3/37 (8)	0/5 (0)	1.0	1/19 (5)	2/23 (9)	1.0
Pulmonary	5/47 (11)	6/37 (16)	1/5 (20)	0.51	4/19 (21)	3/23 (13)	0.68
Renal	4/47 (9)	4/37 (11)	0/5 (0)	0.83	0/19 (0)	4/23 (17)	0.11
Muscular	0/47 (0)	1/37 (3)	0/5 (0)	0.47	0/19 (0)	1/23 (4)	1.0
PNS	2/47 (4)	2/37 (5)	0/5 (0)	1.0	1/19 (5)	1/23 (4)	1.0
CNS	2/47 (4)	0/37 (0)	0/5 (0)	0.56	0/19 (0)	0/23 (0)	-
Haematological	11/47 (23)	8/37 (22)	0/5 (0)	0.76	5/19 (26)	3/23 (13)	0.43
Biological	25/47 (53)	20/37 (54)	2/5 (40)	0.89	10/19 (53)	12/23 (52)	1.0
ESSPRI total score	6.7 (4.7-7.7)	6.0 (4.5-8.0)	8.0 (6.2-8.7)	0.24	5.0 (3.0-6.3)	8.0 (6.0-8.3)	0.01*
ESSPRI domain scores							
Sicca	7.0 (5.0-8.0)	8.0 (5.0-9.0)	9.0 (8.5; 9.5)	0.06	6.0 (5.0-9.0)	8.0 (8.0-9.0)	0.046*
Fatigue	7.0 (5.0-8.0)	7.0 (3.0-8.0)	8.0 (4.0; 9.0)	0.64	5.0 (2.0-8.0)	8.0 (5.0-9.0)	0.09
Pain	5.0 (1.0- 7.0)	5.0 (3.0; 8.0)	8.0 (3.5; 9.5)	0.18	4.0 (2.0-5.0)	7.0 (5.0-9.0)	0.01*
Anti-SSA seropositive (%)	40/47 (85)	32/37 (87)	5/5 (100)	1.00	18/19 (95)	19/23 (83)	0.36
Anti-SSB seropositives (%)	28/47 (60)	22/37 (60)	2/5 (40)	0.78	13/19 (68)	11/23 (48)	0.22
ANA seropositives (%)	38/46 (83)	28/36 (78)	4/5 (80)	0.92	13/19 (68)	19/22 (86)	0.26
RF seropositives (%)	33/46 (72)	25/36 (69)	3/5 (60)	0.89	12/19 (63)	16/22 (73)	0.51
Focal sialoadenitis (%)	36/38 (95)	22/24 (92)	3/5 (60)	0.06	10/12 (83)	15/17 (88)	1.0
IgG (g/L)	14.4 (+/- 5.09)	15.7 (+/- 5.7)	12.8 (+/- 4.99)	0.40	15.9 (+/- 6.98)	14.9 (+/- 4.50)	0.68
C3 (g/L)	0.92 (+/- 0.19)	0.94 (+/-0.23)	1.04 (+/- 0.29)	0.43	0.94 (+/- 0.25)	0.97 (+/- 0.24)	0.73
C4 (g/L)	0.18 (+/- 0.06)	0.18 (+/- 0.06)	0.20 (+/- 0.07)	0.67	0.19 (+/- 0.08)	0.19 (+/- 0.05)	0.98
Type I IFN positive (%)	33/47 (70)	29/37 (78)	2/5 (40)	0.16	15/19 (79)	16/23 (70)	0.73

*= p<0.05; Data are presented as n/n total (%). For continuous data: mean (+/- SD)/median (q1-q2). Comparisons between the mean or median of more than 2 groups were performed using One-way ANOVA or Kruskal-Wallis test. Means or medians of two groups were compared using independent T-test or Mann Whitney U-test. Discrete variables were compared using Chi2 test or Fisher's exact test. ESSDAI is presented as ESSDAI total score as well as presence of activity in each ESSDAI domain. ESSPRI is presented as ESSPRI total as well as domain scores. ANA: Anti-nuclear antibodies, C: complement component, CNS: central nervous system, ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index, ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index, IFN: interferon, IgG: Immunoglobulin G, PNS: peripheral nervous system, RF: rheumatoid factor.

IFN signature, ESSDAI/ESSPRI and patient characteristics

When studying associations between type I IFN signature and ESSDAI scores, only the ESSDAI articular domain score was significantly lower in the type I IFN signature positive group in comparison with the type I IFN signature negative group (0.0 (0.0; 0.0) vs 0.0 (0.0; 2.0), p<0.01). In addition, the ESSPRI total score (6.0

(4.0; 7.7) vs 7.7 (5.2; 8.2); p=0.04), ESSPRI sicca score (7.0 (5.0; 8.0) vs 8.0 (6.0; 9.0); p=0.03) and ESSPRI pain score (4.5 (1.3; 7.0) vs 6.0 (5.0; 8.0); p= 0.02) were also significantly lower in the type I IFN positive group (Table 3). Type I IFN signature positive patients were significantly more often seropositive for anti-SSA (97% vs 60%; p<0.01), anti-SSB (69% vs 32%; p<0.01), ANA (89% vs 58%; p<0.01), RF (81% vs 42%; p<0.01), and had higher titers of IgG (mean 15.7 g/L; SD 5.26 vs 12.5 g/L; SD 4.95, p=0.01) compared to type I IFN signature negative patients. However, no differences in presence of focal sialoadenitis, or levels of complement 3 or 4 were found between these groups (Table 3).

Table 3:
Clinical characteristics stratified on IFN activation

	IFN I neg, n=25	IFN I pos, n=64	p-value
ESSDAI total score	3.0 (0.0-8.0)	3.0 (1.0-7.8)	0.85
ESSDAI activity in each domain			
Constitutional	5/25 (20)	8/64 (13)	0.50
Lymphadenopaty	1/25 (4)	3/64 (5)	1.0
Glandular	2/25 (8)	8/64 (13)	0.72
Articular	7/25 (28)	3/64 (5)	<0.01*
Cutaneous	2/25 (8)	4/64 (6)	1.0
Pulmonary	1/25 (4)	11/64 (17)	0.17
Renal	2/25 (8)	6 /64 (9)	1.0
Muscular	0/25 (0)	1/64 (2)	1.0
PNS	1/25 (4)	3/64 (5)	1.0
CNS	1/25 (4)	1/64 (2)	0.49
Haematological	4/25 (16)	15/64 (23)	0.44
Biological	13/25 (52)	34/64 (53)	0.92
ESSPRI total score	7.7 (5.2-8.2)	6.0 (4.0-7.7)	0.04*
ESSPRI domain scores			
Sicca	8.0 (6.0-9.0)	7.0 (5.0-8.0)	0.03*
Fatigue	8.0 (5.0-9.0)	7.0 (5.0-8.0)	0.26
Pain	6.0 (5.0-8.0)	4.5 (1.3-7.0)	0.02*
Anti-SSA seropositives	15/25 (60)	62/64 (97)	<0.01*
Anti-SSB seropositives	8/25 (32)	44/64 (69)	<0.01*
ANA seropositives	14/24 (58)	56/63 (89)	<0.01*
RF seropositives	10/24 (42)	51/63 (81)	<0.01*
Focal sialoadenitis	16/18 (89)	45/49 (92)	0.66
IgG (g/L)	12.5 (+/- 4.95)	15.7 (+/- 5.26)	0.01*
C3 (g/L)	0.94 (+/- 0.21)	0.93 (+/- 0.22)	0.83
C4 (g/L)	0.19 (+/- 0.07)	0.18 (+/- 0.06)	0.42

*p<0.05; Data are presented as n/n total (%). For continuous data: mean (+/- SD)/median (q1-q3). Independent T-test for continuous, normally distributed variables, Mann Whitney U-test for continuous non-normally distributed variables, Chi2 test or Fisher's exact test for discrete variables ESSDAI is presented as an ESSDAI total scores as well as presence of activity in each ESSDAI domain. ESSPRI is presented as ESSPRI total as well as domain scores. ANA: Anti-nuclear antibodies, C: complement component, CNS: central nervous system, ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index, ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index, IFN: interferon, IgG: Immunoglobulin G, PNS: peripheral nervous system, RF: rheumatoid factor.

Discussion

In this study, 6%, 41% and 53% of the patients with pSS were current smokers, former smokers and never smokers, respectively. Seventy-two percent were type I IFN signature positive. No associations were found between ESSDAI total score or presence of activity in ESSDAI domains and smoking habits or cigarette consumption. A higher cumulative cigarette consumption was associated with higher scores in ESSPRI total and ESSPRI sicca and pain domains. No associations between smoking habits and type I IFN signature positivity was found. Additionally, the type I IFN signature was associated with the presence of autoantibodies and IgG-

levels as well as a negative association to the ESSPRI total, sicca and pain domain scores, and ESSDAI articular domain score.

Several previous studies [11,10,9,25] have shown a negative association between smoking and pSS diagnosis. However, it is unclear whether the reported negative association is due to a local effect of smoking in the exocrine glands or to a more systemic effect. In the current study we investigated whether smoking habits is associated with the phenotypic expression of pSS. We did not find any evidence that previous smoking alters the phenotype of pSS, neither as evaluated by ESSDAI-scores nor by type I IFN signature. Considering the low number of current smokers, caution should be taken when evaluating the results as there is a clear risk of not detecting true differences. However, in this study we noted numerically fewer, namely 40%, type I IFN signature positive patients amongst current smokers vs 78 % in former and 70% in never smokers (p=0.16) which could be investigated in a larger study, especially since cigarette smoking has been shown to suppress IFN I signalling [26].

The higher scores in ESSPRI sicca domain amongst patients with a higher cumulative cigarette consumption (≥8.8 packyears) (p=0.046) does signal a long term effect by smoking which could be explained by a harmful effect on the salivary glands, and not an effect on the disease development of pSS, since long term smoking has previously been shown to be associated to reduced amount and quality of saliva [27]. Furthermore, patients with a higher cumulative smoking exposure (≥8.8 pack-years) scored significantly higher in ESSPRI pain domain (p=0.01) which is in line with previous reports of smoking being a risk factor for chronic pain [28] and might thus not be associated with the development of pSS.

We did not find any association between type I IFN signature and ESSDAI total score in the current cohort although there was a significant lower prevalence of activity in the ESSDAI articular domain amongst type I IFN signature positive patients. Previous studies have shown that the association between the type I IFN signature and the ESSDAI varies between studied cohorts [5-7] . This could be due to the insufficient sensitivity of the ESSDAI to tap systemic inflammation or that the systemic inflammation measured by ESSDAI is not solely driven by type I IFN [29,30].

Concerning symptoms, we found an inverse association between type I IFN signature and ESSPRI total, sicca and pain scores. A negative association between ESSPRI and IFN signature and other proinflammatory cytokines has previously been described [5,31]. A possible explanation for this finding could be that the mechanisms regulating inflammation also affect symptoms such as fatigue and pain as proposed by Howard Tripp et al [31]. There are also reports that IFNα inhibits nociceptive transmission in the spinal cord which could provide an explanation to the negative association between type I IFN signature and the ESSDAI pain domain [32]. Furthermore, there are also reports that treatment with low dose IFNα

improves salivary flow in pSS patients [33], which can be due to an increased expression of the aquaporine-5 gene [34].

Despite the low number of current smokers in this study, we noted a numerically lower frequency of focal sialadenitis amongst current smokers which did not reach statistical significance in the current study, but which is in accordance with previous observations [11,9].

Smoking exposure in this cohort of consecutive patients was mostly long before the study started and the amount of consumed cigarettes varied a lot. Therefore, the population of ever smokers and former smokers was heterogenous. As expected, the number of current smokers was low, making it difficult to draw conclusions about the association to current smoking. Some trends amongst current smokers were noted though, which could be further investigated in a larger study including more current smokers. However, there is a trend of reduced frequency of daily smokers in the general population [35]. Therefore, it might be difficult to include a larger number of current daily smokers in future studies.

Strengths of the current study include the use of consecutive, well-characterised patients in routine clinical care. However, there were several limitations of the study, including the small number of current smokers and heterogenicity concerning cigarette consumption. Retrospectively acquired data on smoking also has limitations due to recall bias. Potential confounders of sicca symptoms, e.g. use of antidepressants or other drugs with anti-cholinergic side effects, were not available. Most patients had long-standing pSS, and we cannot exclude that different patterns may be observed in patients with recent onset of clinical disease.

In conclusion

No significant differences were found between never, former or current smokers in ESSDAI total score or presence of activity in the ESSDAI domains. Of note, current smokers were few in this study making it difficult to draw any firm conclusions regarding this group. Likewise, no difference in type I IFN signature was found by smoking habits or smoking exposure (pack-years). However, patients with a higher cumulative smoking exposure scored significantly higher in ESSPRI total, and ESSPRI sicca and pain domains scores whilst type I IFN signature positive patients scored significantly lower in ESSPRI total, ESSPRI sicca and pain domains and ESSDAI articular domain. In conclusion, there does not seem to be a strong and consistent association between former smoking and disease activity, patient reported symptoms and laboratory signs of systemic inflammation in pSS, at least not in established disease. Previously described associations between IFN signature, presence of autoantibodies and increased IgG-levels were confirmed.

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Competing interests

The authors declare no competing interests.

Ethical statement

The current study was approved by the regional ethical review board for southern Sweden (Lund, Sweden: 2015/311 and 2017/94). All patients gave written informed consent in accordance with the declaration of Helsinki.

Abbreviations

ACR:	American College of Rheumatology
AECG:	American European Consensus Group
ANA:	Anti-nuclear antibodies
Anti-SSA:	anti Sjögren’s syndrome A antibody
Anti-SSB	anti Sjögren’s syndrome A antibody
C3:	Complement component 3
C4:	Complement component 4
cDNA:	complementary deoxyribonucleic acid
CNS:	Central nervous system
EGM:	Extra glandular manifestations
ESSDAI:	EULAR Sjögren’s Syndrome Disease Activity Index
ESSPRI:	EULAR Sjögren’s Syndrome Patient Reported Index
EULAR:	European League Against Rheumatism
HLA:	Human leukocyte antigen
IFN	Interferon
IQR:	Interquartile range
PNS:	Peripheral nervous system
pSS:	Primary Sjögren’s syndrome
RNA:	Ribonucleic acid
RT-PCR:	Reverse transcriptase polymerase chain reaction
SD:	Standard deviation

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Supplementary material

Smoking habits

A. Have you ever been smoking on a regular basis ☐ YES ☐ NO

If YES ☐ cigarettes?

If YES, when did you start? Age: ....years

☐ pipe?

☐ cigars?

B. Have you quit smoking? ☐ YES ☐ NO

If you have quit smoking, when did you quit?

Age: ....years

C. Your current smoking habits .... cigarettes/day

.... gr pipe tobacco/week (1 package = 50 g)

.... cigars/week

D. During the whole period you have been smoking: how much have you been smoking on average?

.... cigarettes/day

.... gr pipe tobacco/week (1 package = 50 g)

.... cigars/week

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