UNIVERSITAT DE BARCELONA

Role Of The Innate Immunity In The Etiopathogenesis of Primary Sjögren Syndrome: Influence Of Viral And Immunogenetic Factors Related To Cell Adhesion Molecules In Systemic Disease And Autoantibody Profile

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DOCTORAL THESIS

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presented by

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DEDICATION

I dedicate this dissertation work to my family. I love you deeply with all my heart. To my husband, Masoud, who has been a source of strength, support, patience, and motivation for me throughout this entire experience. I am truly blessed to have you as my partner in this dance called life. To my daughter, Yasamin, and my son, Alisan, remember all things are possible. Never be afraid to pursue your dreams and goals. You both are precious gifts from the Lord. I love you without measure. To my parents, a special feeling of gratitude to my loving Mom whose words of encouragement and push for tenacity ring in my ears. To my Dad who always told me I could achieve anything I chose to do. To my brother who have never left my side. Thank you all for your patience as I pursued and completed this degree.

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SYNOPSIS

Sjögren Syndrome is a systemic autoimmune disease that affects the exocrine glands, most notably the salivary and lacrimal glands, although it can affect other mucosal surfaces. Inflammation at these sites leads to a spectrum of sicca symptoms, thereby the predominantly symptoms are dryness of the mouth and the eyes. It is not limited to exocrine gland dysfunction; it may involve any other extraglandular systems as well (kidneys, lungs, central and peripheral nervous systems, among others). It is called primary SjS when it exists as an isolated diagnosis, and associated SjS when it co-occurs with another systemic autoimmune disorder. It is characterized by the presence of anti-Ro/La antibodies and/or the presence of focal lymphocytic sialadenitis. SjS is a chronic, systemic autoimmune disease for which no cure currently exists and the management should be organ-specific. The etiopathogenetic factors contributing to SjS remain enigmatic. The development of SjS can be considered as occurring in three stages: 1) an environmental trigger incites autoimmunity against a specific genetic background, b) the autoimmune response becomes chronic due to aberrant regulatory mechanisms within the immune system, and c) lymphoepithelial lesions and subsequent tissue damage arise from persistent inflammation. The prevailing theory, termed 'autoimmune epithelitis,' posits that the epithelial cells of the exocrine glands are the primary sites of inflammation in SjS. According to this model, lymphocytic infiltrates develop in epithelial cells surrounding or invading organs. These epithelial cells act as central players in the autoimmune response by functioning as atypical antigen-presenting cells. Extensive research has been devoted to investigating the role of salivary gland epithelial cells and epithelial cells of the lacrimal glands in SjS. These studies have demonstrated that epithelial cells are capable of orchestrating both innate and acquired immune responses, thereby affirming their crucial role in the disease process. The study of innate immunity in the context of autoimmune diseases like SjS is still in its infancy. Emerging evidence suggests that innate immune components may play a critical role in SjS etiopathogenesis.

In this thesis, we focus on the role of HCV and innate immunity components (pattern recognition receptor SP-D, scavenger receptors CD5 and CD6 and adhesion molecule ALCAM) in SjS etiopathogenesis. Individuals infected with HCV often present sicca symptoms that mimic those of SjS. The virus is known to be associated with autoimmune phenomena and histopathological evidence shows that HCV can be isolated in the salivary glands of infected individuals. SP-D is found in epithelial cells and luminar material in glandular tissues, potentially implicating it in the

pathogenesis of primary SjS. CD5, CD6, and ALCAM/CD166 are expressed in inflamed tissues of SjS, and their SNPs have been linked to other immune-mediated inflammatory diseases. Based on the evidence, we believe that individuals with HCV infection and those with specific SNPs in innate immunity components (SP-D, CD5/CD6, and ALCAM/CD166) may exhibit altered disease expression and immunological profiles in SjS.

Understanding the role of these factors could provide critical insights into the onset and perpetuation of autoimmune responses in SjS. The overarching goal is to advance our understanding of how innate immunity machinery and specific etiopathogenic factors contribute to the complex landscape of SjS, thereby providing new avenues for diagnosis, prognosis, and therapeutic intervention.

Sinopsi

La síndrome de Sjögren és una malaltia autoimmune sistèmica que afecta les glàndules exocrines, sobretot a les glàndules salivals i lacrimals, encara que pot afectar altres superfícies mucoses. La inflamació en aquests llocs desencadena un espectre de símptomes de sicca (queratoconjuntivitis seca), de manera que els símptomes predominants són la sequedat de boca i d'ulls. No es limita a la disfunció de les glàndules exocrines, sinó que també pot afectar altres sistemes extraglandulars (ronyons, pulmons, sistema nerviós central i perifèric, entre altres). Es denomina SjS primari quan existeix com a diagnòstic aïllat, i SjS secundari quan es associat (coexisteix) amb un altre trastorn autoimmune sistèmic. Es caracteritza per la presència d'anticossos anti-Ro/La i/o la presència de sialoadenitis limfocítica focal. El SjS es una malaltia autoimmune sistèmica crònica per a la qual ara per ara encara no existeix cura i on el tractament de la qual ha de ser específic per a l'òrgan. Els factors etiopatogènics que contribueixen al SjS continuen sent enigmàtics. Pot considerar-se que el desenvolupament del SjS es produeix en tres etapes: 1) un desencadenant ambiental incita a l'autoimmunitat contra un determinat antecedent genètic, b) la resposta autoimmune es cronifica a causa de mecanismes reguladors anòmals dins del sistema immunitari, i c) les lesions limfoepitelials i el dany tissular conseqüent sorgeixen de la inflamació persistent. La teoria que més s'ha imposat, denominada "epitelitis autoimmune", sosté que les cèl·lules epitelials de les glàndules exocrines són el principal focus d'inflamació en el SjS. Segons aquest enfocament, es desenvolupen limfòcits infiltrats en cèl·lules epitelials que envolten o envaeixen els òrgans. Aquestes cèl·lules epitelials tenen un paper central en la resposta autoimmune perquè funcionen com a cèl·lules presentadores d'antígens atípiques. S'han dut a terme nombroses recerques sobre el paper de les cèl·lules epitelials de les glàndules salivals i de les glàndules lacrimals en el SjS. Aquests estudis han demostrat que les cèl·lules epitelials estan capacitades per a crear respostes immunitàries tant innates com adquirides, afirmant així el seu paper crucial en el desenvolupament de la malaltia. L'estudi de la resposta immunitària innata en el context de malalties autoimmunitàries com el SjS es troba encara en els seus inicis. Cada vegada hi ha més proves que afirmen que els components immunitaris innats poden exercir un funció fonamental en l'etiopatogènia del SjS.

En aquesta tesi, ens centrem en el paper del VHC i els components de la immunitat innata (receptors de reconeixement de patrons SP-D, receptors scavenger CD5 i CD6, i la molècula d'adhesió ALCAM) en l'etiopatogènia del SjS. Les persones infectades

pel VHC acostumen a presentar símptomes de sicca (queratoconjuntivitis seca) que s'assemblen als del SjS. Se sap que el virus està associat a fenòmens autoimmunes i les proves histopatològiques demostren que el VHC pot aïllar-se en les glàndules salivals dels individus infectats. El SP-D es troba en les cèl·lules epitelials i en el material luminar dels teixits glandulars, la qual cosa podria implicar-lo en la patogènesi del SjS primari. Els CD5, CD6 i ALCAM/CD166 s'expressen en els teixits inflamats del SjS, i els seus SNP s'han relacionat amb altres malalties inflamatòries inmunomediades. Basant-nos en aquestes proves, creiem que els individus amb infecció pel VHC i aquells amb SNPs en components de la immunitat innata (SP-D, CD5/CD6, i ALCAM/CD166) poden mostrar una manifestació alterada de la malaltia i perfils immunològics en el SjS.

La comprensió del funcionament d'aquests factors podria aportar informació significativa sobre l'inici i la perpetuació de les respostes autoimmunes en el SjS. L'objectiu general és avançar en la nostra recerca sobre com l'estructura de la immunitat innata i els factors etiopatogènics específics contribueixen al complex marc del SjS, i proporcionar així noves vies per al diagnòstic, el pronòstic i la intervenció terapèutica.

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LIST OF ABREVIATIONS

ACA - Anticentromere antibodies

ACR - American College of Rheumatology

AECG - American–European Consensus Group

ALCAM - Activated Leukocyte Cell Adhesion Molecule

AMA - Antimitochondial antibodies

ANA - Antinuclear antibodies

anti-dsDNA - Antibodies against double strand deoxyribonucleic acid

anti-La/SSB - Anti–Sjögren's-syndrome-related antigen B autoantibodies

anti-RNP - Antibodies against ribonucleoprotein

anti-Ro/SSA - Anti–Sjögren's-syndrome-related antigen A autoantibodies

APA - Antiphospholipid antibodies

BAFF - B cell-activating factor

BCR - B cell receptor

BD - Behçet's disease

CAMs - Cell adhesion molecules

CD - Crohn disease

CLRs - C-type lectin receptors

Collectins - Collagen-containing C-type lectins

CRDs - Carbohydrate recognition domains

CyA - Cyclosporin A

DAMPs - danger-associated molecular patterns

EBV - Epstein–Barr virus

ESR - Erythrocyte sedimentation rate

ESSDAI - EULAR Sjögren's Syndrome Disease Activity Index

EULAR - European League Against Rheumatism

FLS - Focal lymphocytic sialadenitis

GCs - Glucocorticoids

GWAS - Genome-wide association studies

HCV - Hepatitis C virus

HHV6 - Human herpesvirus 6

HLA-DR - HLA-antigen D related

HTLV1 - Human T cell lymphotropic virus 1

IBD - Inflammatory bowel diseases

IFN - Interferon

Ig - Immunoglobulin

Ig-domains - Immunoglobulin domains

IgG - Immunoglobulin G

IgM - Immunoglobulin M

IgSF - Immunoglobulin Superfamily

ILD - Interstitial lung disease

ITAM-like - Immunoreceptor tyrosine-based activation motif–like

KL-6 - Krebs von den Lungen-6

LSG - Labial salivary gland

MALT - Mucosa-associated lymphoid tissue

MBL - Mannan-binding lectin

MC - Mixed Cryoglobulinemia

MCTD - Mixed connective tissue disease

mIgs - Monoclonal gammopathy

MRI - Magnetic Resonance Imaging

MS - Multiple sclerosis

NK – Natural killer

NLRs - Nucleotide-binding domain and leucine-rich repeat-containing receptors

PAMPs - Pathogen-associated molecular patterns

PBC - Primary Biliary Cholangitis

PRRs - Pattern recognition receptors

Ps - Psoriasis

RA - Rheumatoid arthritis

rfhSP-D - Human SP-D

RNA - Ribonucleic acid

SGECs - Salivary gland epithelial cells

SjS - Sjögren syndrome

SjS-HCV - SjS concomitant with HCV

SLE - Systemic lupus erythematosus

SNPs - Single nucleotide polymorphisms

SP-A - Surfactant protein-A

SP-D - Surfactant protein-D

SRCR-SF - Scavenger receptor cysteine-rich superfamily

SSc - Systemic sclerosis

STING - Stimulator of interferon genes

TCR - T cell receptor

TFH - Follicular helper T

TH - T helper

TLRs - Toll-like receptors

UC - Ulcerative colitis

UTR - Untranslated regions

V-domains - Variable domains

BACKGROUND

Sjögren Syndrome (SjS) represents a systemic autoimmune condition primarily targeting the exocrine glands, most notably the salivary and lacrimal glands. However, it can also manifest in the nose, upper respiratory tract, oropharynx, and in females, the vaginal area. Inflammation at these sites leads to a spectrum of sicca symptoms, predominantly causing mucosal dryness in the oral and ocular regions. Hendrik Sjögren was the pioneer in identifying that the classical sicca symptoms transcended mere glandular dysfunction, and consequently, the term Sjögren Syndrome (SjS) was coined (1).

The nomenclature designates it as 'primary SjS' when SjS exists as an isolated diagnosis, and 'associated SjS' when it co-occurs with another systemic autoimmune disorder. The usage of these terms remains a subject of ongoing discussion, lacking standardized guidelines for their appropriate employment. The term 'associated SjS' is generally reserved for cases featuring concurrent systemic autoimmune illnesses, such as rheumatoid arthritis (RA), systemic sclerosis (SSc), or systemic lupus erythematosus (SLE), but is seldom applied to cases with organ-specific autoimmune diseases like autoimmune thyroiditis, primary biliary cholangitis, or autoimmune hepatitis (1).

1.Epidemiology

The incidence and prevalence rates of SjS exhibit substantial variability, contingent upon both the study methodology and the diagnostic criteria employed (2). Studies utilizing the 1993 European Classification Criteria reported a 12-fold increase in pooled prevalence rates compared to those adopting the 2002 American-European Consensus Group (AECG) criteria. Moreover, the prevalence figures derived from population-based epidemiological studies appear marginally lower than those calculated for the overall population (2). Incidence rates of SjS oscillate between 3 and 11 cases per 100,000 persons (3, 4), with prevalence rates ranging from 0.01% to 0.72%. (5, 6). It is plausible that a subset of asymptomatic individuals remains undiagnosed.

Upon disease presentation, SjS displays a distinct epidemiological profile, potentially facilitating early diagnosis. Primarily, the disease disproportionately affects females, exhibiting the most skewed gender distribution among all systemic autoimmune diseases; a nearly 10:1 female-to-male ratio has been noted in a comprehensive data study involving over 14,000 SjS patients (7). Secondly, although SjS can manifest at any age, the peak diagnosis occurs between the ages of

30 and 50. Pediatric cases are rare, and the gender disparity is less pronounced in children than in adults (8).

In individuals diagnosed with systemic autoimmune diseases, the proportion who also have concomitant SjS varies by condition: 14–18% in cases with SLE (9, 10), 7–17% with RA and 12% with SSc (1, 11, 12).

2. Clinical manifestations

Sjögren Syndrome (SjS) is a multifaceted autoimmune condition that spans a range from isolated sicca symptoms to broad systemic involvement, referred to as extraglandular manifestations (13). While sicca symptoms primarily impact the quality of life and induce localized mucosal complications, systemic involvement significantly influences disease prognosis. SjS patients may manifest an array of extraglandular symptoms, either at the onset of the disease or during its subsequent course (14, 15).

2.1. Sicca symptoms

2.1.1. Oral dryness

Xerostomia, the subjective sensation of oral dryness, stands as a hallmark symptom in SjS, occurring in conjunction with xerophthalmia in over 95% of cases. Accompanying oral symptoms may encompass soreness, food adhesion to mucosal surfaces, and dysphagia. Reduced salivary flow complicates oral functions such as speaking and eating and predisposes patients to localized infections, dental caries, and periodontal diseases (13).

2.1.2. Ocular dryness

Xerophthalmia, the subjective experience of ocular dryness, is reported in upwards of 90% of patients. This symptomatology can include itchiness, grittiness, and may extend to soreness, photosensitivity, eye fatigue, and decreased visual acuity. Reduced tear secretion exacerbates chronic ocular irritation and epithelial damage (keratoconjunctivitis sicca), heightening the risk of ocular infections (13). Extreme light sensitivity (photophobia) to the point of pain or inability to open the eyelids may signal the onset of corneal ulcers. Reduced tear film production is particularly evident in contact lens wearers, as they often begin to exhibit symptoms of lens intolerance (13).

2.1.3. Additional Sicca Manifestations

Involvement of other mucosal surfaces leads to a broad array of clinical presentations. Pharyngeal dryness may trigger pruritus, the sensation of a foreign body within the pharynx, and dysphagia, affecting around 30% of SjS patients (1). Respiratory mucosal dryness may culminate in epistaxis or an irritable cough, while cutaneous dryness (xerosis) stemming from reduced sweat gland activity can induce pruritus and/or alopecia. Vaginal dryness may present with symptoms such as pruritus and dyspareunia (16, 17).

Approximately 30% of patients may also experience intermittent inflammatory swelling of the major salivary glands, specifically the parotid and submandibular glands (13).

2.2. Systemic involvement

Sjögren's Syndrome (SjS) is not limited to exocrine gland dysfunction; it may involve extraglandular systems as well. These systemic manifestations can span multiple bodily systems, including but not limited to the skin (cutaneous), joints (articular), lungs (pulmonary), heart (cardiovascular), kidneys (nephro-urological), nervous system, and blood (hematological) (Table 1) (1).

TABLE 1. Systemic manifestations in SjS. *(reference 1)*

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).

These symptoms may either be initial presentations or develop later in the disease course. In cases where systemic symptoms precede or overshadow sicca symptoms, the condition is sometimes termed "occult SjS" or "non-sicca onset of SjS" (18).

To assess disease activity, the EULAR SjS Disease Activity Index (ESSDAI) was developed, comprising 12 organ-specific 'domains' (10 clinical and 2 analytical). The ESSDAI scores disease activity on a scale from 0 to 123 (19) (Table 2).

TABLE 2. EULAR SjS disease activity index (ESSDAI) *(reference 19)*

2.1) Constitutional domain

Domain	Activity level	Description
Constitutional Exclusion of fever of infectious origin and voluntary weight loss	No=0	Absence of the following symptoms
	Low=3	Mild or intermittent fever (37.5−38.5°C)/night sweats and/or involuntary weight loss of 5–10% of body weight
	Moderate=6	Severe fever (>38.5°C)/night sweats and/or involuntary weight loss of >10% of body weight

2.2) Lymphadenopathy and lymphoma domain

Domain	Activity level	Description
Lymphadenopathy and lymphoma Exclusion of infection	No=0	Absence of the following features
	Low=4	Lymphadenopathy ≥1 cm in any nodal region or ≥2 cm in inguinal region
	Moderate=8	Lymphadenopathy ≥2 cm in any nodal region or ≥3 cm in inguinal region, and/or splenomegaly (clinically palpable or assessed by imaging)
	High=12	Current malignant B-cell proliferative disorder

2.3) Glandular domain

Domain	Activity level	Description
Glandular Exclusion of stone or infection	No=0	Absence of glandular swelling
	Low=2	Small glandular swelling with enlarged parotid (≤3 cm), or limited submandibular (≤2 cm) or lachrymal swelling (≤1 cm)
	Moderate=4	Major glandular swelling with enlarged parotid (>3 cm), or important submandibular (>2 cm) or lachrymal swelling (>1 cm) Absence of glandular swelling

2.4) Articular domain

Domain	Activity level	Description
Articular Exclusion of osteoarthritis	No=0	Absence of currently active articular involvement
	Low=2	Arthralgias in hands, wrists, ankles and feet accompanied by morning stiffness (>30 min)
	Moderate=4	1–5 (of 28 total count) synovitis
	High=6	≥6 (of 28 total count) synovitis

2.5) Cutaneus domain

Domain	Activity level	Description
Cutaneous Rate as 'No activity' stable long-lasting features related to damage	No=0	Absence of currently active cutaneous involvement
	Low=3	Erythema multiforma
	Moderate=6	Limited cutaneous vasculitis, including urticarial vasculitis, or purpura limited to feet and ankle, or subacute cutaneous lupus
	High=9	Diffuse cutaneous vasculitis, including urticarial vasculitis, or diffuse purpura, or ulcers related to vasculitis

2.6) Pulmonary domain

Domain	Activity level	Description
Pulmonary 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=5	Persistent cough due to 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=10	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=15	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%

2.7) Renal domain

Domain	Activity level	Description
Renal 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/day, no haematuria, no leucocyturia, no acidosis or long-lasting stable proteinuria due to damage
	Low=5	Evidence of mild active renal involvement, limited to tubular acidosis without renal failure or glomerular involvement with proteinuria (between 0.5 and 1 g/day) and without haematuria or renal failure (GFR ≥60 mL/min)
	Moderate=10	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/day and without haematuria or renal failure (GFR ≥60 mL/min) or histological evidence of extra-membranous glomerulonephritis or important interstitial lymphoid infiltrate
	High=15	Highly active renal involvement, such as glomerular involvement with proteinuria >1.5

2.8) Muscular domain

Domain	Activity level	Description
Muscular Exclusion of weakness due to corticosteroids	No=0	Absence of currently active muscular involvement
	Low=6	Mild active myositis shown by abnormal EMG, MRI* or biopsy with no weakness and creatine kinase (N≤CK≤2N)
	Moderate=12	Moderately active myositis proven by abnormal EMG, MRI* or biopsy with weakness (maximal deficit of 4/5), or elevated creatine kinase (2N<CK≤4N),
	High=18	Highly active myositis shown by abnormal EMG, MRI* or biopsy with weakness (deficit ≤3/5) or elevated creatine kinase (>4N)

*We decided to add this item not included in the initial version since the value of this examination for the diagnosis of myositis was not clear until recently. EMG, electromyogram.

2.9) PNS domain

Domain	Activity level	Description
PNS *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=5	Mild active PNS involvement, such as pure sensory axonal polyneuropathy shown by NCS or trigeminal (V) neuralgia *Proven small fibre neuropathy*
	Moderate=10	Moderately active PNS involvement shown by NCS, such as axonal sensory–motor neuropathy with maximal motor deficit of 4/5, pure sensory neuropathy with presence of cryoglobulinemic vasculitis, ganglionopathy with symptoms restricted to mild/moderate ataxia, inflammatory demyelinating polyneuropathy (CIDP) with mild functional impairment (maximal motor deficit of 4/5 or mild ataxia) Or cranial nerve involvement of peripheral origin (except trigeminal (V) neuralgia)
	High=15	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

*We decided to add this item not included in the initial version since the link between this entity and SS was not clear until recently.

2.10) Central nervous system domain

Domain	Activity level	Description
CNS 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
	Moderate=10	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=15	Highly active CNS features, such as cerebral vasculitis with cerebrovascular accident or transient ischaemic attack, seizures, transverse myelitis, lymphocytic meningitis, multiple sclerosis-like syndrome with motor deficit

2.11) Biological domain

Domain	Activity level	Description
Biological	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)

2.12) Haematological domain

Domain	Activity level	Description
Haematological	No=0	Absence of autoimmune cytopenia
For anaemia, neutropenia, and thrombopenia, only auto-immune cytopenia must be considered	Low=2	Cytopenia of autoimmune 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)
Exclusion of vitamin or iron deficiency, drug-induced cytopenia	Moderate=4	Cytopenia of autoimmune 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=6	Cytopenia of autoimmune origin with neutropenia (neutrophils<500/mm3), and/or or anaemia (haemoglobin<8 g/dL) and/or thrombocytopenia (platelets<50 000/mm3)

General Symptoms

General manifestations commonly observed in SjS include fever, weight loss, and fatigue (1).

Articular Involvement

Synovitis or arthritis is frequent in SjS patients but usually leads to less joint damage compared to RA. The condition in SjS is more likely to be relapsing-remitting in nature (15), but causes less joint erosion and is more relapsing and remitting than in RA (15). Subclinical synovitis can often be detected through musculoskeletal ultrasonography (20).

Cutaneous Involvement

Skin manifestations include cutaneous vasculitis and annular erythema.

Cutaneous vasculitis may present as flat purpura in patients with hypergammaglobulinemia and palpable purpura associated with cryoglobulinemic vasculitis (15, 21), while annular erythema presents as an erythematous eruption with a ring-shaped appearance and usually associated with anti-Ro antibodies.

Pulmonary Involvement

About 16% of SjS patients have pulmonary involvement, although it is rarely clinically significant. Patients are at higher risk of developing interstitial lung diseases and commonly display certain autoantibodies and lymphopenia (22).

Nephro-Urological Involvement

The kidneys may be affected through lymphocytic infiltration causing interstitial nephritis or through immune complex-mediated membranous or membranoproliferative glomerulonephritis (23). Some renal complications may be clinically silent, such as distal renal tubular acidosis.

Both the peripheral and central nervous systems can be involved. Peripheral neuropathies are found in about 10% of SjS patients, with sensory neuropathy being the most frequent (24), Central nervous system involvement is less common, estimated at 3% of cases, and can include myelitis, aseptic meningitis, and cerebral lesions resembling those in multiple sclerosis (MS) (15).

Other Complications

Other complications include Raynaud's phenomenon, serositis, and protein-losing enteropathy, among others (25).

Systemic involvement in SjS is variable and can significantly impact prognosis and quality of life. Accurate diagnosis and timely management are crucial for optimizing patient outcomes.

3. Laboratory abnormalities

3.1. Serological abnormalities

In Sjögren's Syndrome (SjS), similar to other systemic autoimmune diseases, laboratory abnormalities can be prevalent and, in some cases, might even be the initial clinical manifestation. Serological markers such as polyclonal hypergammaglobulinemia and elevated erythrocyte sedimentation rate (ESR) are commonly used in the diagnosis and monitoring of the disease.

3.1.1. ESR

Elevated ESR levels are a common feature in SjS patients, with over 60% having ESR values exceeding 50 mm in the first hour. Elevated ESR often correlates with high circulating protein levels, particularly hypergammaglobulinemia (26).

3.1.2. Gamma globulins

High levels of gamma globulins are another frequent laboratory abnormality. Polyclonal hypergammaglobulinemia occurs in approximately half of SjS patients, resulting from lymphocyte hyperactivity. Monoclonal gammopathy is found in 22% of primary SjS patients—making SjS the systemic autoimmune disease with the highest prevalence of this condition. The most common type of band detected is mIgGκ (1). Hypogammaglobulinemia may also be present but is less frequent.

Additionally, raised levels of β2-microglobulin have been reported in about one-third of patients (1, 27). These abnormalities in the gammaglobulins are part of the biological domain in the ESSDAI.

3.2. Hematological abnormalities

Hematological abnormalities are common in SjS, affecting approximately one-third of patients. The most common cytopenias include:

- Anemia: 20% of patients
- Leukopenia: 16% of patients
- Thrombocytopenia: 13% of patients

Bicytopenia is present in 10%, and pancytopenia is rare, seen in only 1% of patients (1).

The type of anemia is usually normocytic, and leukopenia may result from lymphopenia and/or neutropenia. These cytopenias are particularly common in patients who test positive for anti-Ro/SSA or anti-La/SSB antibodies.

Though these cytopenias are often asymptomatic, they can become clinically significant in some cases. They are closely associated with systemic disease and can be predictive of poor outcomes (1, 24, 28-30). Hematological abnormalities are included as one of the 12 domains in the ESSDAI.

These laboratory and hematological abnormalities are vital components in the diagnosis and management of SjS. Recognizing and understanding these markers can guide clinicians in both the diagnosis and long-term monitoring of this complex disease.

3.3. Immunological Anomalies

Autoantibodies serve as the principal serological indicators in autoimmune disorders, manifesting up to two decades prior to the formal diagnosis of primary SjS (31, 32).

3.3.1. Antinuclear Autoantibodies

Antinuclear antibodies (ANAs) target the nuclear and cytoplasmic components of human cells. As these antibodies are detected in approximately 80% of SjS patients, they function as valuable biomarkers for the diagnosis of SjS, particularly in those exhibiting sicca symptoms (1).

3.3.2. Anti-Ro/SSA and anti-La/SSB Autoantibodies

Autoantibodies against Ro/SSA target Ro52 and Ro60 proteins, which associate with small RNA molecules. These antibodies are identified in up to 70% of SjS patients, although the prevalence may differ based on the detection methodology employed. Approximately 12–20% of anti-Ro/SSA-negative patients may still exhibit specific antibodies against Ro52 and Ro60. Current literature advocates for individualized assessment of anti-Ro52 and anti-Ro60 due to their association with different SjS clinical phenotypes (33-35). Autoantibodies against La/SSB target the La/SSB protein involved in various facets of RNA metabolism, including viral RNA binding. They are identified in nearly half of SjS patients and often co-occur with anti-Ro/SSA antibodies. The concurrent presence of these antibodies is strongly associated with an increased likelihood of ANA-positivity and systemic disease manifestation (36, 37).

A subset of SjS patients, constituting between 2.3% and 7%, exhibit isolated anti-La/SSB antibodies without accompanying anti-Ro/SSA antibodies (1).

3.3.3. Rheumatoid factor

This autoantibody targets the Fc fragment of IgG and is present in half of the SjS patient population. Despite its prevalence, the rheumatoid factor is not part of the existing classification criteria for SjS due to its low specificity, given its occurrence across multiple autoimmune diseases (19).

3.3.4. Cryoglobulins

Cryoglobulins are immunoglobulins that precipitate at temperatures below 37°C in vitro. Nearly one in ten SjS patients show the presence of circulating cryoglobulins, predominantly of mixed type (comprising both IgG and IgM) (38). These serve as critical prognostic markers, signifying a higher likelihood of systemic involvement, B-cell lymphoma, and mortality (24, 28, 39, 40). Therefore, cryoglobulin detection is imperative both at the initial diagnosis and throughout disease management.

3.3.5. Hypocomplementemia

Characterized by reduced levels of complement components C3 and/or C4, hypocomplementemia is evident in 10–25% of SjS patients. Similar to cryoglobulins, hypocomplementemia is correlated with adverse outcomes like lymphoma and mortality (28). Circulating monoclonal immunoglobulins, or monoclonal gammopathy of undetermined significance, are detected in up to one-fifth of SjS patients, with mIgGκ being the most prevalent subtype (28, 41, 42).

3.3.6. Additional Autoantibodies
3.3.6.1. Anti-dsDNA Antibodies

Anti-double-stranded deoxyribonucleic acid antibodies (anti-dsDNA) and anti-Sm antibodies primarily serve as diagnostic markers for SLE, and are typically absent in patients with primary SjS. However, emerging research suggests that elevated titers of anti-dsDNA antibodies in primary SjS patients may indicate a potential progression towards SLE (43).

3.3.6.2. Anti-RNP Antibodies

Autoantibodies against ribonucleoproteins (anti-RNP) are observed in 8–28% of cases, potentially encompassing both primary and secondary forms of SjS. An evaluation for Mixed Connective Tissue Disease (MCTD) is warranted in these instances (43).

3.3.6.3. Anticentromere Antibodies

The detection of anticentromere antibodies (ACA) in SjS patients raises suspicion for a possible evolution towards limited SSc. It is prudent to screen for ACA in all SjS patients exhibiting Raynaud's phenomenon, especially those with elevated ANA titers and negative anti-Ro/anti-La status (43).

3.3.6.4. Antiphospholipid Antibodies

Various studies have investigated the prevalence of antiphospholipid antibodies (APA) in primary SjS patients, documenting a prevalence ranging from 2% to 33%. However, the presence of APA is generally deemed an autoimmune epiphenomenon without substantial clinical implications (43).

3.3.6.5. Antimitochondrial Antibodies

Antimitochondrial antibodies (AMA) were initially identified in 1965 in patients with Primary Biliary Cholangitis (PBC), where they occur in approximately 95% of cases. Given the frequent overlap between PBC and primary SjS, the identification of AMA in a primary SjS patient strongly implies the coexistence of PBC (43).

4. Lymphoma Development in SjS Patients

Patients diagnosed with SjS exhibit an elevated risk of developing lymphoma compared to both the general population and those with other autoimmune diseases. Prior to the year 2000, studies showed a standardized incidence ratio (SIR) ranging from 9 to 44, which has since declined to between 5 and 15 in studies post-2000 (19).

Marginal zone lymphomas, with a particular emphasis on mucosa-associated lymphoid tissue (MALT) lymphomas, constitute the most prevalent histological subtypes seen in SjS patients (19)

While the salivary glands serve as the primary site for lymphoma manifestation, other mucosal regions such as the stomach, lungs, and eyes can also be affected.

Persistent parotid enlargement is the most frequent clinical symptom of lymphoma in SjS patients. Discriminating between benign and malignant parotid enlargement can be challenging. However, some clinical markers can assist in differentiation: benign enlargement is often bilateral and variable in size, whereas lymphoma-associated swelling is more commonly unilateral, fixed, and occasionally firm to the touch (1).

Ultrasonography, inclusive of Doppler studies, and Magnetic Resonance Imaging (MRI) may serve as additional resources for differentiating between benign and malignant parotid enlargement (1).

Major clinical predictors for lymphoma development include sustained salivary gland swelling, lymphadenopathy, and palpable purpura, which is primarily associated with cryoglobulinemia. Recent data from an international cohort study, Big Data Sjögren, indicates that MALT lymphomas in SjS are linked to an overall favorable prognosis, demonstrating a 5-year survival rate of nearly 90% (44).

5. Diagnosis of SjS

The objective of diagnosing SjS is to provide a comprehensive assessment of the subjective symptoms related to dryness. Various tests are available to both confirm and quantify this dryness.

5.1. Lacrimal function tests

These tests aim to evaluate and quantify glandular dysfunction within the lacrimal glands. Prominent ocular evaluations include Schirmer's tests and corneal surface analyses using specific dyes like fluorescein and lissamine green. These dyes stain degenerated or dead cells, commonly referred to as corneal stainings (1).

5.2. Salivary function tests

Two key oral evaluations measure salivary gland function: salivary flow rates and salivary gland scintigraphy. Imaging techniques like ultrasonography and MRI are primarily utilized to assess common complications associated with SjS, such as infections and lymphoma (19).

5.3. Histopathology

Labial Salivary Gland (LSG) biopsy is frequently employed for the diagnosis of primary SjS. It plays a pivotal role in both the American-European Consensus Group classification Criteria (45) and the ACR/EULAR criteria (46). The most distinguishing histopathological feature in affected salivary glands is the presence of focal lymphocytic sialadenitis (FLS), with a sensitivity and specificity of greater than 80% (47). The presence of one or more germinal center-like structures in the salivary glands correlates with a heightened risk of lymphoma development (48, 49).

5.4. Classification criteria

In 2016, ACR and EULAR established an international set of classification criteria for primary Sjögren's syndrome (Table 3). Developed for use in individuals exhibiting signs or symptoms suggestive of SjS, these criteria consist of a weighted sum of five key parameters: anti-SSA/Ro antibody positivity and focal lymphocytic sialadenitis with a focus score ≥1 foci/4 mm^2, each scoring 3 points; and an abnormal Ocular Staining Score ≥5 (or van Bijsterveld score ≥4), a Schirmer's test result ≤5 mm/5 min, and an unstimulated salivary flow rate ≤0.1 mL/min, each scoring 1 point. Individuals with a total score of ≥4 meet the criteria for primary SjS. Sensitivity and specificity in the final validation cohort were remarkably high: 96% (95% CI 92%–98%) and 95% (95% CI 92%–97%), respectively (46).

TABLE 3. 2016 ACR-EULAR Classification Criteria for primary SjS *(reference 46)*

The classification of SjS applies to any individual who meets the inclusion criteria[1], does not have any condition listed as exclusion criteria[2], and who has a score ≥ 4 when summing the weights from the following items:

Item	Weight / Score
Labial salivary gland with focal lymphocytic sialadenitis and focus score ≥ 1.	**3**
Anti-SSA (Ro) +	**3**
Ocular staining score ≥ 5 (or van Bijsterveld score ≥ 4) on at least one eye	**1**
Schirmer ≤ 5 mm/5 min on at least one eye	**1**
Unstimulated whole saliva flow rate ≤ 0.1 ml/min	**1**

1.Inclusion criteria: these 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 recurring sensation of sand or gravel in the eyes? 3) Do you use tear substitutes more than 3 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 help in swallowing dry food?); or suspicion of SjS from ESSDAI questionnaire (at least one domain with positive item).

2.Exclusion criteria: Prior diagnosis of any of the following conditions would exclude diagnosis of SjS and participation in SjS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests: History of head and neck radiation treatment. Active Hepatitis C infection (with positive PCR). Acquired immunodeficiency síndrome. Sarcoidosis. Amyloidosis. Graft versus host disease. IgG4-related disease .

6. Prognosis of SjS

6.1. Risk Stratification

Patients with SjS can be divided into two distinct categories: those with elevated risks for systemic complications or lymphoma and those with relatively low risks. Approximately 25%–30% of SjS patients fall into the high-risk category, warranting more frequent medical follow-ups. For these individuals, clinical assessments every 3–6 months are strongly recommended (1).

6.2. Monitoring

Regular ESSDAI score monitoring by the physician is essential for risk assessment and management. Two pivotal risk factors not encapsulated in the ESSDAI score are high focus scores (≥3) and the presence of germinal centers in the salivary glands. When multiple risk factors are present, more frequent follow-ups are necessitated to manage the elevated risk for systemic complications or lymphoma (1).

If lymphoma is suspected, further diagnostic and treatment pathways should be decided upon in consultation with oncologists (50).

6.3. Ocular and Oral Health

For the preservation of oral health, patients should undergo bi-annual dental examinations aimed at prophylactic treatment and early recognition of tooth damage, which is mainly due to salivary deficiency (51). Although the ocular surface is infrequently affected by irreversible damage in SjS, mandatory ophthalmologist visits are required in the most severe and treatment-resistant cases (1).

7. Treatment of SjS

SjS is a chronic, systemic autoimmune disease for which no cure currently exists. Despite its identification over a century ago, the therapeutic landscape has remained relatively unchanged in recent years (52, 53).

In 2010, the European League Against Rheumatism (EULAR) initiated the first evidence and consensus-based international guidelines for managing SjS, covering both topical and systemic treatments (54).

7.1. Organ-Specific Management

Management should be organ-specific, especially for primary SjS. For localized, glandular manifestations, topical oral treatments like saliva substitutes and artificial tear drops for the eyes are recommended. In severe cases, options such as topical non-steroidal anti-inflammatory drugs, topical corticosteroids, topical cyclosporine A (CyA), and serum tear drops may be considered. Systemic treatment options for dryness include oral muscarinic agonists like pilocarpine and cevimeline.

7.2. Systemic Treatment

Traditional systemic treatment for SjS involves the use of glucocorticoids (GCs) and immunosuppressive agents, borrowed from protocols for similar systemic diseases like SLE or vasculitis (55). Low-dose GCs are used for moderate systemic activity, while high-dose GCs and second-line therapies like cyclophosphamide, azathioprine, methotrexate, leflunomide, and mycophenolate are reserved for refractory or severe cases. Systemic therapies are not recommended for managing dryness, chronic pain, or fatigue (53).

7.3. Emerging Therapies

Currently, direct and indirect B-cell targeted therapies offer the most promise for treating systemic manifestations of primary SjS. Research is ongoing to develop other potential therapeutic approaches, including T-cell co-stimulation, cytokine-based therapies, intracellular pathways, and gene therapies. The next decade may bring revolutionary changes to the treatment landscape for primary SjS (52).

8. Aetiopathogenetic Mechanisms of SjS

The etiopathogenetic factors contributing to SjS remain enigmatic. Various potential mechanisms—incessant activation, defective regulation, or inherent defects within the immune system—have been proposed. The development of SjS can be considered as occurring in three stages (56):

1. An environmental trigger incites autoimmunity against a specific genetic background.
2. The autoimmune response becomes chronic due to aberrant regulatory mechanisms within the immune system.
3. Lymphoepithelial lesions and subsequent tissue damage arise from persistent inflammation.

8.1. Autoimmune Epithelitis Theory

The prevailing theory, termed 'autoimmune epithelitis,' posits that the epithelial cells of the exocrine glands are the primary sites of inflammation in SjS (57). According to this model, lymphocytic infiltrates develop in epithelial cells surrounding or invading organs. These epithelial cells act as central players in the autoimmune response by functioning as atypical antigen-presenting cells (Figure 1).

FIGURE 1. **The pathogenesis of autoimmune epitelitis as a potential explanation for SjS**
(reference 1)
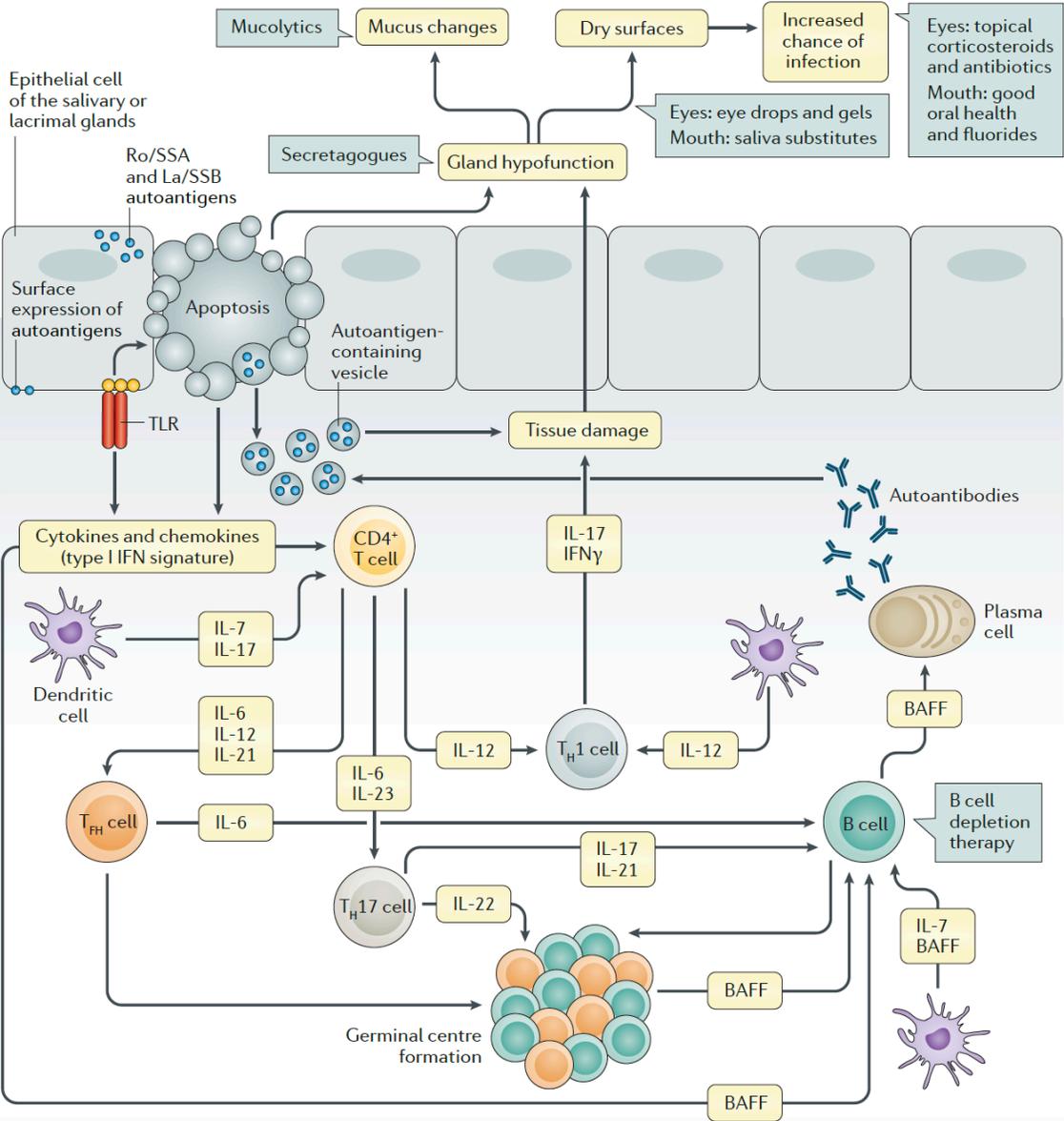
Immune-competent molecules, such as Toll-like receptors (TLRs) are constitutively expressed by salivary gland epithelial cells (SGECs). Activation of TLR signalling in gland epithelium causes the production of autoantigens, the upregulation of immune-competent molecules (chemokines and cytokines), apoptosis and epithelial hypofunction. Autoantigens can be released from SGECs and presented to immune cells. CD4+ T cells differentiate into follicular helper T (TFH) cells, which increase B cell survival. Interaction between SGECs and B cells promotes B cell differentiation. Potential treatments are highlighted in green boxes. BAFF, B cell-activating factor; IFN, interferon; TH, T helper.

Though autoimmune epithelitis is the most widely supported theory, alternative explanations exist. Some studies suggest the involvement of a neuroendocrine mechanism, focusing on the role of hormones and neuropeptides in modulating the function of exocrine glands (58). This could account for the presence of severe sicca symptoms in some SjS patients who exhibit limited or no inflammatory histopathological features (59).

8.2. Role of Epithelial Cells in Immune Responses

Extensive research has been devoted to investigating the role of salivary gland epithelial cells (SGECs) and epithelial cells of the lacrimal glands in SjS. These studies have demonstrated that epithelial cells are capable of orchestrating both innate and acquired immune responses, thereby affirming their crucial role in the disease process (60, 61).

9. Pathogenic Factors Influencing SjS

9.1. Genetic Factors

The genetic underpinnings of SjS are assumed to be as multifaceted as those of other autoimmune disorders like SLE and RA, both of which have over 100 known genetic associations. In contrast, the genetic landscape of SjS is less explored. Familial clustering reveals that approximately 35% of SjS patients have relatives with either SjS or another autoimmune condition (62). Furthermore, individuals with a twin sibling who has SjS face a 662-fold increased risk of developing the disease (63).

9.1.1. Role of HLA Molecules

Genome-wide association studies have identified robust links between SjS and Human Leukocyte Antigen (HLA) class I, II, and III regions. Specifically, multiple associations have been found with HLA class II molecules, notably HLA-antigen D related (HLA-DR) and HLA-DQ loci (64, 65). Some of these HLA class II alleles correlate with the production of autoantibodies in SjS but not with other clinical manifestations (65).

9.1.2. Expression Quantitative Trait Loci (eQTLs)

Integration of genetic and transcriptional data has led to the identification of expression quantitative trait loci (eQTLs) in various HLA class I and II loci like HLA-A, HLA-C, HLA-

DRB6, HLA-DPB1, and HLA-DQA1 (66). These findings suggest that the risk alleles may not only alter the function of HLA molecules, such as peptide binding and antigen presentation, but could also impact their expression levels (1).

9.1.3. Other Genetic Loci

Additionally, several other loci linked to both innate and adaptive immune processes show significant association with SjS. These include genes implicated in type I and II IFN signaling (e.g., IRF5, IL12A, and STAT4), NF-κB signaling (e.g., TNIP1 and TNFAIP3), lymphocyte trafficking (e.g., CXCR5), and the activation and differentiation of antibody-producing cells (e.g., BLK) (64).

9.1.4. Unanswered Questions

Despite these findings, it remains unclear how these SjS-associated genetic variants modify normal biological processes or contribute to the disease. For instance, eQTLs have also been identified in IL12A, BLK, and TNIP1 alleles, hinting that these variants may influence the expression levels of the associated proteins (64). Also, the specific subsets of immune cells affected by these genetic variants are not yet well-understood. While BLK expression is largely restricted to B-cells, other associated genes are expressed in a variety of immune cells, including NK cells, monocytes, dendritic cells, T cells, and B cells (64). Understanding these genetic effects and their impact on immune function is crucial for unraveling the disease mechanisms of SjS (1).

9.2. Viral environmental Factors

The interplay between intrinsic (genetic) and extrinsic (environmental) factors is a hallmark in the onset and progression of SjS. Notably, viruses have consistently been considered pivotal environmental agents in SjS etiopathogenesis. Salivary glands, which are primarily affected in SjS, are also known reservoirs for latent viral infections (1, 67).

9.2.1. Various Viruses and Their Roles

Viruses from the Herpesviridae family, such as Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV6), have been under the spotlight for their potential role in SjS. However, these viruses

are ubiquitous, making it difficult to establish a direct causal relationship. Conflicting data and their high prevalence in the general population further complicate the matter (68-71). Retroviruses like human T-cell lymphotropic virus 1 (HTLV1) deserve special mention. In areas of Japan endemic for HTLV1, significantly elevated levels of HTLV1-specific antibodies were noted in SjS patients. This suggests a potential role for retroviruses in triggering autoimmune responses that lead to SjS (72).

9.2.2 Hepatitis C Virus (HCV)

Hepatitis C Virus (HCV) is particularly significant when it comes to understanding the etiopathogenesis of SjS (36).

It is a small, positive-polarity, single-stranded RNA virus from the Hepacivirus genus of the Flaviviridae family. The virus's RNA genome encodes a polyprotein that is crucial for its lifecycle and pathogenicity (73, 74) (Figure 2). The HCV core is a highly conserved protein that makes up the viral nucleocapsid and plays role in pathogenesis (75, 76).

FIGURE 2. Schematic illustration of the Hepatitis C virus *(reference 77)*
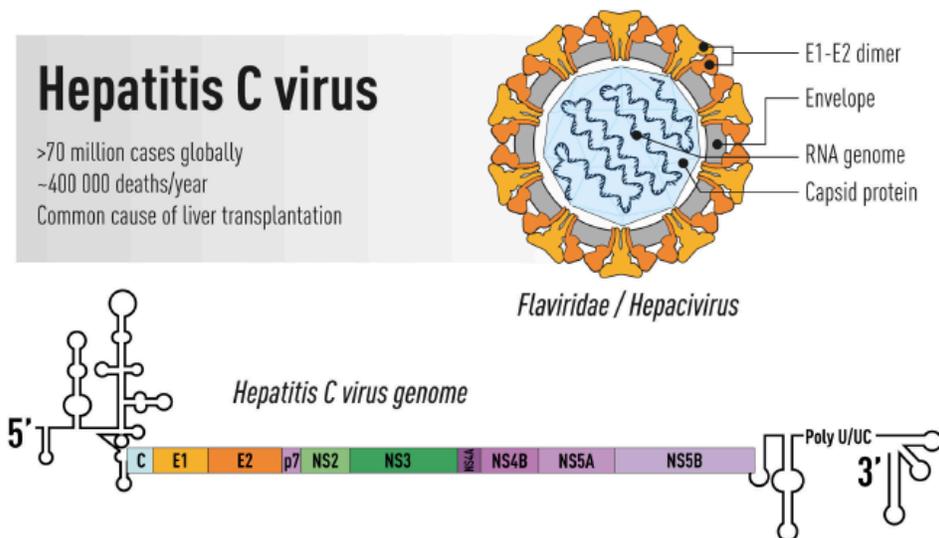
Top right: the virus particle containing an RNA genome and the viral envelope glycoproteins E1 and E2 exposed on the surface. Bottom: the viral genome encoding a large polyprotein that is cleaved into multiple structural and non-structural proteins with 5' and 3' terminal untranslated regions.

9.2.3. Regional Variability

Studies in Mediterranean countries indicate that patients with SjS have a higher prevalence (14%) of chronic HCV infection compared to the general population. This prevalence appears to differ between Mediterranean and non-Mediterranean countries, emphasizing the role of geographical and perhaps even cultural factors in the disease etiology (78).

9.2.4. HCV's Role in Autoimmune Features

Besides its hepatic manifestations, chronic HCV infection is linked to a variety of non-hepatic conditions like pulmonary fibrosis, cutaneous vasculitis, and glomerulonephritis, among others (79), Importantly, cryoglobulinaemia, a condition associated with autoimmune features, has increasingly been recognized in the context of chronic HCV infection (80, 81).

9.2.5. Salivary Gland Pathology

Histological examinations have demonstrated that HCV is frequently found in SGECs of SjS patients with chronic sialadenitis. Remarkably, a much higher frequency of focal lymphocytic sialadenitis (FLS) is observed in SjS patients who are also HCV-positive (57%) compared to those without HCV (5%) (1, 82).

9.2.6. Molecular Links: La Protein

The human La protein serves as a fascinating molecular link between HCV and SjS. Not only is it a principal autoantigen in SjS, but it also plays a crucial role in HCV translation, suggesting shared immune-mediated etiopathogenic mechanisms (36, 83).

9.2.7. Unresolved Questions and Future Directions

Despite progress, many questions remain unanswered. Understanding how viruses, especially HCV, activate autoimmunity is a subject of ongoing research. The role of pattern-recognition receptors in viral-mediated autoimmunity is also gaining interest. Finally, the temporal aspect adds another layer of complexity; autoantibodies have been discovered in patients, decades before SjS diagnosis (1, 31, 84).

Through an integrated understanding of these factors, we can hope to unravel the intricate genetic and environmental tapestry that culminates in SjS.

10. Innate Immunity

Innate immunity has been increasingly implicated in autoimmune diseases, serving as the frontline defense and also as a complex modulator of immune homeostasis. Unlike adaptive immunity, which is characterized by its antigen specificity, the innate immune system is an ancient, universal form of host defense. It plays a crucial role in identifying and eliminating non-self or altered-self molecules either directly or by triggering an adaptive immune response (85-87).

The innate immune system operates at two distinct levels:

1. **Physical and Chemical Barriers**: The skin, mucosal tissue, blood-brain barrier, and chemical elements (e.g., fatty acids, pH, enzymes, and complement system) serve as the host's initial defenses against invading pathogens (85-87).

2. **Innate Immune Cells**: This subsystem in vertebrates encompasses a variety of cells, including monocytes, neutrophils, macrophages, dendritic cells, natural killer (NK) cells, mast cells, eosinophils, and basophils. These cells carry out non-specific immune defense and surveillance (87-89).

The innate immune system relies on Pattern Recognition Receptors (PRRs) for pathogen identification. These receptors bind unique pathogen-associated molecular patterns (PAMPs), which are conserved structures vital for pathogenic survival but not found in the host (89-93).

Activation of PRRs triggers intracellular signaling cascades, resulting in the transcriptional expression of proinflammatory cytokines, type I interferons, and other antiviral proteins. These molecules coordinate the immune response aimed at eliminating pathogens and infected cells (89).

PRRs are categorized into multiple classes:

1. **Toll-Like Receptors (TLRs)**: Primarily involved in detecting bacterial and viral components (94).

2. **RIG-I-like Receptors**: Recognize viral RNA (95),

3. **NLRs (Nucleotide-binding domain and leucine-rich repeat-containing receptors)**: Respond mainly to intracellular pathogen (96).

4. **C-type Lectin Receptors (CLRs)**: Target fungal and other pathogens (97).

5. **cGAMP Synthase and STING**: Involved in detecting DNA pathogens (98, 99).

10.1. Innate Immunity in SjS

In SjS, a comprehensive understanding of innate immunity is vital given its implications in the disease's etiopathogenesis. The dysregulation of innate immune components, particularly PRRs like TLRs, has been noted in SjS patients, indicating a potential role in the disease's initiation and progression.

The study of innate immunity in the context of autoimmune diseases like SjS is still in its infancy. Emerging evidence suggests that innate immune components may play a critical role in these diseases' etiopathogenesis. As research advances, understanding the intricate interactions between innate and adaptive immunity may provide new avenues for treatment and disease management.

10.2. Collectins

Collectins represent a vital group of pattern-recognition molecules within the innate immune system. Known for their multifaceted roles, they primarily function in pathogen recognition via their calcium-dependent carbohydrate recognition domains (CRDs) (100).

Collectins are complex oligomers composed of trimeric subunits. While most of these subunits are homotrimers, heterotrimers are also present. Each subunit is made up of:

1. **N-terminal cysteine-rich domain**: This short segment, containing 7-28 amino acid residues, is responsible for multimerization via disulfide bridging (100, 101).
2. **Collagen-like domain**: Consists of Gly-X-Y triplet repeats, where X and Y can be any amino acids.
3. **Coiled-Coil Segment**: A short section capable of forming coiled-coil helices.
4. **C-terminal CRD**: Also known as the Carbohydrate Recognition Domain, responsible for pathogen recognition (100, 101).

As of now, nine collectins have been identified:

1. Mannan-binding lectin (MBL)
2. Three bovine serum collectins
3. Conglutinin
4. CL-43 and CL-46
5. Lung surfactant proteins SPA and SP-D
6. Collectin kidney 1 (CL-K1, also called CL-11)

7. Collectin liver 1 (CL-L1, also called CL-10)

8. Collectin placenta 1 (CL-P1, also called CL-12)

The functions of collectins extend beyond merely recognizing pathogens. They are also implicated in (100):

1. **Microbial Aggregation and Neutralization**: Collectins can form clusters of microbes, thereby making it easier for immune cells to identify and eliminate them.

2. **Opsonisation**: This process facilitates the recognition and phagocytosis of pathogens by immune cells.

3. **Complement Activation**: Collectins can trigger the complement system, a biochemical cascade that helps clear pathogens from an organism.

4. **Modulation of Inflammatory Responses**: They play a role in controlling the level and type of immune responses, thus preventing overreactions that could be detrimental (100).

An understanding of the role of collectins in innate immunity provides valuable insights into autoimmune conditions like SjS. Dysregulation in the activity or expression of collectins could be a key factor in the onset and progression of such disease.

As our understanding expands, it may offer novel therapeutic avenues in the management of autoimmunity and infections. Targeting collectins could also be instrumental in designing more efficient vaccines and treatments for a variety of infectious diseases.

10.2.1. SP-D

Surfactant protein-D (SP-D), a pivotal member of the collectin family, has a multifaceted role in the innate immune system, from neutralizing pathogens to modulating inflammation and allergies. Intriguingly, it also bridges the innate and adaptive immune systems through its receptor's capability to interact with major IgG subclasses (93, 102) (Figure 3).

FIGURE 3. Structure of surfactant proteins A (SP-A) and SP-D *(reference 103)*
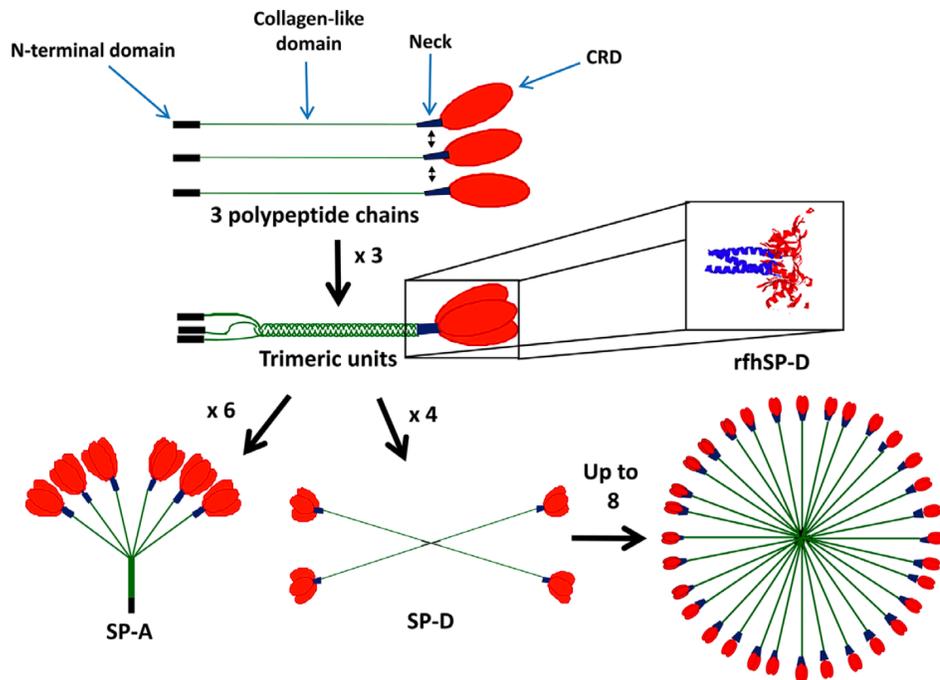
SP-A and SP-D contain four domains: the N-terminal domain (black), collagen-like domain (green), neck region (blue) and carbohydrate recognition domain (CRD) (red). SP-A and SP-D form functional trimers and can then further oligomerize into an octadecamericlike structure for SP-A and a dodecameric cruciform-like structure, which can further assemble into 'stellate multimers' for SP-D. Also shown is the crystal structure of the recombinant fragment of human SP-D (rfhSP-D). rfhSP-D is formed of the CRD, neck and 8x Gly Xaa Yaa repeats of the collagen-like region.

Although predominantly synthesized by alveolar type-II cells in the lungs (104, 105) SP-D has been detected in a variety of extrapulmonary tissues, ranging from heart, kidneys, and brain, to smaller organs like salivary glands and pancreas (106-108). Similar to other collectins, SP-D can eliminate microbes through various mechanisms, such as aggregation, opsonization, and complement activation. It also plays a role in the modulation of adaptive immune responses (100).

10.2.1.1. Polymorphism and Its Impact

A notable polymorphism in the SP-D gene (Met11Thr) significantly affects serum SP-D levels and its multimerization capacity (109, 110). The Thr11 variant has been linked to lower SP-D levels in serum, reduced microbial binding, and diminished oligomerization (110).

10.2.1.2. SP-D serum levels and autoimmunity

10.2.1.2.1 SSc with Interstitial lung disease

Elevated SP-D serum levels have been observed in interstitial lung diseases (ILD) and SSc associated with ILD, potentially reflecting disease severity (111-113). A recent large-scale study found that elevated SP-D levels, in conjunction with anti-topoisomerase I antibodies, could predict ILD occurrence in SSc with an 80% accuracy (114). However, SP-D levels did not correlate with mortality or progression of lung impairment in these patients, creating a debate over its utility as a diagnostic or prognostic marker (115-117).

A sharp decrease in serum SP-D levels post-treatment with cyclophosphamide and prednisolone was observed to predict positive treatment response (118).

10.2.1.3. Comparative Study with KL-6: Sensitivity vs Specificity

When compared with another biomarker, KL-6, SP-D showed higher sensitivity but lower specificity for ILD in SSc patients (116). Both KL-6 and SP-D significantly correlated with fibrosis scores but not with ground-glass opacities on high-resolution CT, raising questions about their ability to reflect radiographic activity of ILD (114).

10.2.1.4. The Multifaceted Role and Future Directions

SP-D serves as an immune workhorse with roles beyond just pathogen clearance. Its varied functions, its involvement in both innate and adaptive immunity, and its potential as a clinical marker make it a compelling subject for future research. Further studies could clarify the controversies surrounding its clinical utility, thereby refining our approach to diagnosing and treating conditions like ILD and SSc.

10.3. Scavenger Receptors CD5 and CD6

CD5 and CD6 are type I transmembrane glycoproteins and members of the scavenger receptor cysteine-rich superfamily (SRCR-SF). This superfamily is renowned for its functional diversity in innate immunity, distinguished by the presence of the highly conserved SRCR domains (119). Each of these receptors consists of three extracellular SRCR domains, designated SRCR1, SRCR2, and SRCR3, followed by a transmembrane domain and a cytoplasmic tail specialized for phosphorylation and intracellular signaling but devoid of enzymatic activity (119).

While CD5 and CD6 are primarily expressed on all types of T cells and the B1a cell subset, they are also present in reduced amounts on other immune cells such as macrophages, dendritic cells, and NK cells (120, 121). Both receptors play critical roles in modulating lymphocyte activation and differentiation, often through physical association with T-cell receptors (TCR) and B-cell receptors (BCR). They act as co-receptors, impacting cellular responses upon antigen recognition (122). Recent studies have unveiled the capability of CD5 and CD6 to interact with pathogen-associated molecular patterns (PAMPs) from bacterial, fungal, viral, or parasitic origins (119).

The CD5 and CD6 genes are located in close proximity on human chromosome 11q12.2 and the analogous region in mouse chromosome 19. With just 11 exons and covering a 24.5 KB region, CD5 lies 82 kb in the 3' direction to CD6. The structural and functional similarities between these two receptors suggest that they likely originated from a common ancestral gene (123, 124) (Figure 4).

FIGURE 4. Genomic location and arrangement of CD5 and CD6 *(reference 119)*
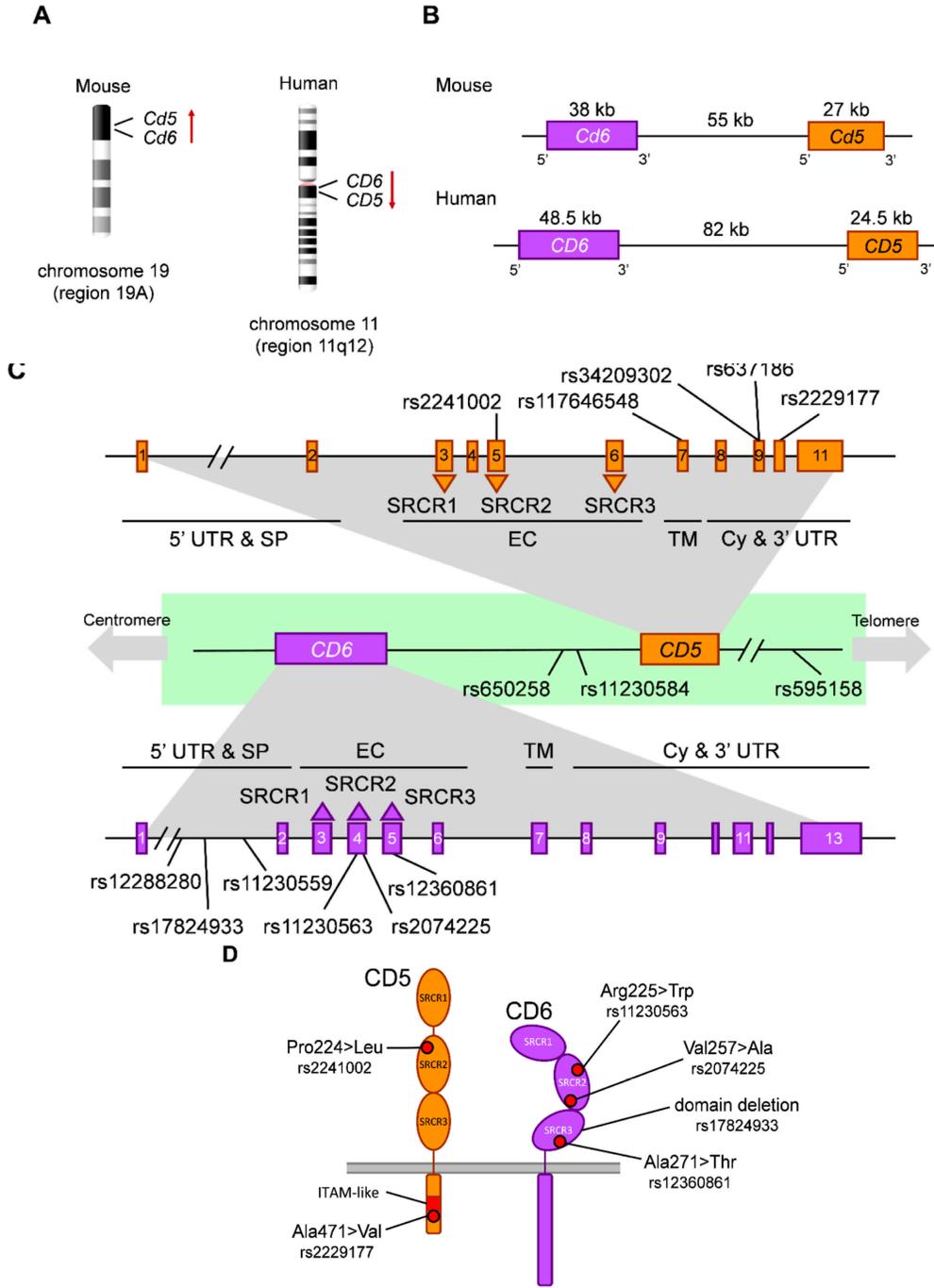
(A) Chromosome location of mouse and human genes coding for CD5 and CD6. Red arrows indicate 5' to 3' orientation. (B) Size, orientation and intergenic distance regarding CD5 and CD6 in mouse and human. (C) Exon/intron organization, protein coding regions and location of relevant SNPs in CD5 and CD6. (D) Structure of membrane CD5 and CD6 showing the impact of relevant SNPs. UTR, untranslated region; EC, extracellular region; Cy, cytoplasmic region; SRCR, scavenger receptor cysteine-rich domain; ITAM-like, immunoreceptor tyrosine-based activation motif–like.

CD5 and CD6 serve as adaptable, multifunctional co-receptors in the immune system, affecting a range of processes from lymphocyte activation to interactions with pathogens. Given their important roles in immune modulation and their versatile expression profiles, further studies on these receptors could provide valuable insights into the complexities of both innate and adaptive immune responses.

10.3.1. CD5 Polymorphism in Autoimmunity

The role of CD5 single nucleotide polymorphisms (SNPs) has become a subject of interest for their potential impact on autoimmune and neoplastic diseases. These genetic variations can serve as markers for susceptibility to these disorders and may even modify disease outcomes.

10.3.1.1. Rheumatoid Arthritis

Genome-wide association studies (GWAS) have identified a CD5 polymorphism (rs595158) that is associated with susceptibility to RA (125).

10.3.1.2. Lupus Nephritis

In SLE, certain CD5 alleles—specifically rs2241002C (Pro224) and rs2229177C (Ala471)—have been found to be associated with lupus nephritis, a severe manifestation of SLE (126). The rs2241002C-rs2229177C haplotype (Pro224-Ala471) is overrepresented among SLE patients with nephritis, supporting the notion that this variant may have reduced immunomodulatory properties.

10.3.1.3. Inflammatory Bowel Diseases: Crohn's and Ulcerative Colitis

Recent studies in patients with inflammatory bowel diseases (IBD) have also shown links between CD5 polymorphism and disease outcomes. In Crohn's disease (CD), specific variations in CD5 are associated with disease location (rs2241002CC) and the need for biological therapies (rs2241002C-rs2229177T haplotype; Pro224-Val471). In ulcerative colitis (UC), certain CD5 variants are linked to poor prognosis (rs2241002T-rs2229177T haplotype; Leu224-Val471) (127).

10.3.1.4. Utility of CD5 polymorphisms

These findings suggest that the role of CD5 genetic variations may differ depending on disease-specific etiopathogenic factors (119).

CD5 polymorphisms may serve as vital markers for assessing disease susceptibility and course in a range of autoimmune conditions. These polymorphisms can have a significant impact on disease outcomes, offering insights into potential therapeutic targets and providing a more comprehensive understanding of disease pathogenesis. Further studies are needed to validate these associations and elucidate the underlying mechanisms.

10.3.2. CD6 Polymorphism in Autoimmunity

CD6 single SNP have been identified as influential factors in several immune-mediated inflammatory disorders, including MS, psoriasis (Ps), and Behçet's disease (BD) (128).

10.3.2.1. Multiple Sclerosis

A meta-analysis consolidating six genome-wide association studies (GWAS) has indicated CD6 as a significant risk locus for MS (128). Particularly, the CD6 rs17824933 SNP was identified as a risk marker, with the rs17824933G allele being associated with greater susceptibility to MS in European cohorts. This finding was corroborated by gene-specific and independent cohort studies (129, 130). Subsequent studies have discovered additional risk alleles, such as rs2074225T (Val257), and risk haplotypes, such as rs11230563T-rs2074225T (Trp225 Val257). These variations have been associated with lower CD6 expression in various lymphocyte subsets (129). While rs17824933 did not prove to be a risk factor in an African American cohort, the SNP rs11230563C (Arg225) was identified as a risk marker for MS (131). No association between CD6 SNPs and MS risk was found in an Asian cohort, although a related intronic allele was associated with neuromyelitis optica (132).

10.3.2.2. Other Inflammatory Diseases

In addition to MS, CD6 polymorphisms have been associated with increased severity in primary SjS in European cohorts (133) and increased risk of BD in a Chinese Han population (134). CD6 variation has also been implicated in IBD, which aligns with recent observations on its association with disease location and prognosis (119).

10.3.2.3. CD6 Polymorphisms as Clinical Predictors

CD6 polymorphisms have proven to be significant factors in the predisposition and severity of various autoimmune disorders. The range of diseases impacted by these genetic variations underscores the crucial role CD6 plays in the immune system. As with CD5, these polymorphisms offer the potential for targeted therapies and personalized treatment approaches for immune-mediated diseases. Further research is necessary to elucidate the precise mechanisms by which these genetic variations contribute to disease pathogenesis.

10.4. Cell Adhesion Molecules

The architecture of multi-cellular tissues largely depends on cell adhesion molecules (CAMs), which serve as biological "glue." However, rather than providing strong physical bonds, CAMs facilitate the cellular sensing required for intricate tissue patterning. These molecules are part of four primary CAM families: cadherins, IgSF (Immunoglobulin Superfamily), selectins, and integrins, with IgSF standing out as the most expansive and diverse group (135).

The Immunoglobulin Superfamily (IgSF) is known for its characteristic Ig-fold: a structure composed of anti-parallel beta-sheets that are sandwiched together. This large and diverse family includes members that serve various functions. In addition to serving as cell adhesion molecules, IgSF proteins are involved as antigen receptors, growth factor receptors, and molecules that provide either costimulatory or inhibitory signals (135).

IgSF proteins are unique in that they mediate calcium-independent cell-cell adhesion. They recognize a variety of ligands—both homophilic (same type) and heterophilic (different types). While they do not form adhesions as robust as those mediated by cadherins or integrins, IgSF proteins play a crucial role in refining, enhancing, and regulating these adhesive interactions (136).

10.4.1. ALCAM (Activated Leukocyte Cell Adhesion Molecule)

Activated Leukocyte Cell Adhesion Molecule (ALCAM) is a transmembrane glycoprotein that is part of a subgroup within the Immunoglobulin Superfamily (IgSF). It consists of five extracellular immunoglobulin-like domains, a transmembrane region, and a variable-length cytoplasmic tail. These domains enable homophilic (ALCAM-ALCAM) as well as heterophilic

(ALCAM-CD6) interactions, the latter of which occurs particularly through the three membrane-proximal constant (C2) domains (137) (Figure 5).

In contrast to other CAMs, ALCAM has been specifically associated with a variety of biological processes ranging from leukocyte activation to tumor progression. As part of the IgSF, ALCAM contributes to fine-tuning cellular interactions, similar to the previously discussed CD5 and CD6 molecules. However, unlike CD5 and CD6, which are primarily expressed on lymphocytes, ALCAM has a broader range of expression and is involved in several biological processes beyond the immune system, serves multiple roles in immune regulation, cellular adhesion, and tissue organization.

Apart from its adhesion functions, ALCAM serves as a marker of cellular identity. It is typically found at junctional regions where cells make contact, signifying its role in epithelial, neuronal, stromal, and mesenchymal tissues (135).

Other roles in which ALCAM could be implicated include: role in tissue organization, immune response, disease progression, hematopoiesis, axon pathfinding and neuronal migration (138) (139) since it is expressed in multiple cell types such as hematopoietic stem cells, cancer stem cells, central nervous system cells, and epithelial crypts of the intestine, among others (140) (139).

ALCAM is subject to various regulatory mechanisms, including transcription, alternative splicing, and membrane trafficking. It also involves multiple binding partners and undergoes proteolytic processes that collectively determine its adhesion capabilities and localization within the cell (135).

The ALCAM gene is situated on human chromosome 3 (3q13.11). Transcriptional regulation involves CpG-rich sequences and multiple motifs, including canonical Sp1 and functional p65 NF-κB (141-143).

10.4.1.1. Association with Disease

ALCAM's heterophilic interactions with the CD6 lymphocyte receptor have been implicated in the pathogenesis of MS, Ps, BD, and IBD (128) (128, 133, 134, 144-148).

Overexpression of ALCAM has also been observed in epithelial lesions in primary SjS (149-151).

10.4.2. Future Perspectives

ALCAM plays a multifaceted role in cellular function, going beyond its primary role as an adhesion molecule to being a marker of cellular identity and a key player in disease pathogenesis. Its widespread expression and involvement in numerous cellular processes make it a fascinating subject for further study, particularly in the context of inflammatory diseases and potential targeted therapies.

FIGURE 5. ALCAM regulation for tunable cell adhesion *(reference 135)*
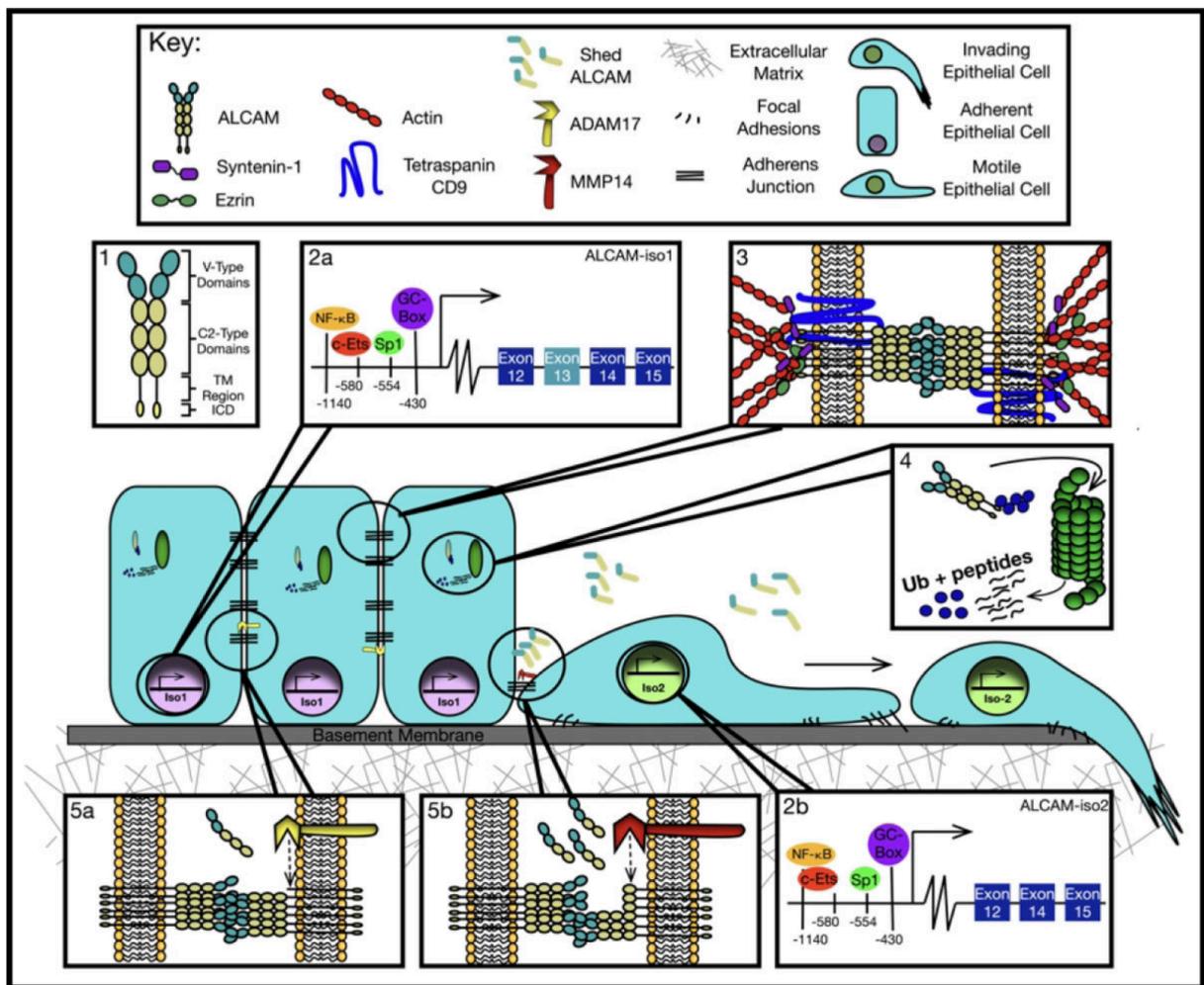
(1) ALCAM is composed of five Ig-like domains, two V-type domains and three C2-type domains, a transmembrane (TM) region, and a small intracellular domain (ICD). ALCAM gene expression (2a/b) is regulated by the promoter elements NF-κB, Ets, Sp1, and a GC-box upstream of the translation start site (TSS). This molecule associates with the tetraspanin CD9 to enhance homotypic ALCAM-ALCAM

interactions and facilitate clustering. It also associates with the actin cytoskeleton to strengthen cell adhesion through adaptors syntenin-1 and ezrin (3). ALCAM protein stability is regulated by CHIP mediated ubiquitination and subsequent proteasomal degradation (4). Alternative splicing affects the proteolytic susceptibility of ALCAM (4). ADAM17 proteolysis of iso1 promotes high cell-cell adhesion through low basal shedding (5a), while MMP14 proteolysis of iso2 enables cell motility through high basal shedding and disruption of ALCAM-ALCAM interactions (5b).

Summary

Aims and Scope

The primary objective of this doctoral thesis is to delve deeper into understanding the etiopathogenesis of SjS with a focus on the role of innate immunity components. Specifically, the work aims to examine the influence of HCV as an environmental-viral factor, along with innate immunity components like pattern recognition receptor SP-D, scavenger receptors CD5 and CD6, and adhesion molecules ALCAM/CD166.

Rationale for Selected Factors

1. **HCV**: The HCV is highlighted for its putative role in SjS for three main reasons:
   - Individuals infected with HCV often present clinical symptoms that mimic those of SjS, including dryness (sicca symptoms).
   - The virus is known to be associated with autoimmune phenomena, particularly cryoglobulinemia, which negatively impacts the prognosis of SjS.
   - Histopathological evidence shows that HCV can be isolated in the salivary glands of infected individuals, and the resulting lymphocytic infiltration is indistinguishable from that seen in SjS.

2. **Innate Immunity Components**: The elements SP-D, CD5/CD6, and ALCAM/CD166 were selected based on the following:
   - **SP-D** is found in epithelial cells and luminar material in glandular tissues, potentially implicating it in the pathogenesis of primary SjS.
   - **CD5, CD6, and ALCAM/CD166** are expressed in inflamed tissues of SjS, and their SNPs have been linked to other immune-mediated inflammatory diseases (IMIDs).

HYPOTHESIS

Hypothesis

Based on the evidence, we hypothesize that individuals with HCV infection and those with specific SNPs in innate immunity components (SP-D, CD5/CD6, and ALCAM/CD166) may exhibit altered disease expression and immunological profiles in SjS.

Significance of the Study

Understanding the role of these factors could provide critical insights into the onset and perpetuation of autoimmune responses, especially in SjS. Given the progress made in our understanding of genetic variants and their functionality in autoimmune diseases, this thesis aims to contribute to the growing body of knowledge, potentially paving the way for more targeted treatment options.

The overarching goal is to advance our understanding of how innate immunity machinery and specific etiopathogenic factors contribute to the complex landscape of SjS, thereby providing new avenues for diagnosis, prognosis, and therapeutic intervention.

OBJECTIVES

GENERAL OBJECTIVE.

The main general objective of this thesis is to analyze the impact of HCV and innate immune molecules (SP-D, CD5/CD6, ALCAM/CD166) on disease expression and immunological profile of SjS.

SPECIFIC OBJECTIVES.

The main specific objectives of each of the three studies were the following:

First article.
How hepatitis C virus modifies the immunological profile of Sjögren syndrome: analysis of 783 patients. *Arthritis Res Ther 2015;17:250*

- To analyze the prevalence of HCV in a large cohort of SjS patients.

- To analyze and compare the main clinical profile of HCV-SjS patients vs non-HCV-SjS patients.

- To analyze and compare the main immunological profile of HCV-SjS patients vs non-HCV-SjS patients.

- To analize the association of HCV genotypes and SjS features.

- To analize the influence of HCV on the SjS-related autoantibody profile.

Second article.
Etiopathogenic Role of Surfactant Protein D in the Clinical and Immunological Expression of Primary Sjögren Syndrome.
J Rheumatol 2015;42:111-8.

- To analyze the prevalence of SP-D SNPs in SjS and healthy controls.

- To analize the relationship between SP-D genotypes (Met11/Met11, Met11/Thr11, and Thr11/Thr11) the main epidemiological, clinical, and immunological characteristics of primary SjS.

- To analize the association between SNPs and serum levels of SP-D.

- To analyze serum SP-D levels in SjS patients and analize the association of SP-D levels and the main epidemiological, clinical, and immunological characteristics of primary SjS.

Alleles: *SFTPD rs721917*

Third article.

Gene Variation at Immunomodulatory and Cell Adhesion Molecules Loci Impacts Primary Sjögren's Syndrome. *Front Med (Lausanne)* 2022;9:822290.

- To analize the prevalence of each individual SNPs of *CD5 CD6* and *CD166/ALCAM* in healthy controls and patients with primary SjS.

- To analize the main epidemiological, clinical and immunological characteristics of pSS patients.

- To analyze the association of each individual SNPs *CD5, CD6* and *CD166/ALCAM* with the main epidemiological, clinical, and immunological characteristics of primary SjS.

- To analyze the association of *CD5/CD6* and *CD166/ALCAM* SNPs with the presence or absence of anti-Ro/La antibodies.

- To analyze the association of *CD5/CD6* and *ALCAM/CD166* SNPs with the main epidemiological, clinical, and immunological characteristics of primary SjS.

*Alleles:
CD5 rs2241002, CD5 rs2229177
CD6 rs17824933, CD6 rs11230563, CD6 rs12360861
CD166/ALCAM rs6437585
CD166/ALCAM rs579565
CD166/ALCAM rs1044243

REPORT FROM THE THESIS DIRECTOR

The current doctoral thesis is made up of 3 scientific articles, of which, Hoda Gheitasi is first co-author in 2 (1st and 3rd article) and 11th in the 2nd article.

All articles were published in international journals ISI (International Scientific Indexing) with IF and within the 1st and 2nd quartiles. More detail information regarding contribution of Hoda is detailed in the next pages.

1

Arthritis Res Ther 2015;17:250

How hepatitis C virus modifies the immunological profile of Sjögren syndrome: analysis of 783 patients
Pilar Brito-Zerón, **Hoda Gheitasi***, Soledad Retamozo, Albert Bové, María Londoño, Jose-Maria Sánchez-Tapias, Miguel Caballero, Belchin Kostov, Xavier Forns, Srini V. Kaveri and Manuel Ramos-Casals

2

J Rheumatol 2015;42:111-8.

Etiopathogenic Role of Surfactant Protein D in the Clinical and Immunological Expression of Primary Sjögren Syndrome
María José Soto-Cárdenas, Myriam Gandía, Pilar Brito-Zerón, Maria Teresa Arias, Noelia Armiger, Albert Bové, Xavier Bosch, Soledad Retamozo, Miriam Akasbi, Marta Pérez-De-Lis, **Hoda Gheitasi**, Belchin Kostov, Roberto Pérez-Alvarez, Antoni Siso-Almirall, Francisco Lozano, and Manuel Ramos-Casals

3

Front Med (Lausanne) 2022;9:822290.

Gene Variation at Immunomodulatory and Cell Adhesion Molecules Loci Impacts Primary Sjögren's Syndrome
Sergi Casadó-Llombart, **Hoda Gheitasi***, Silvia Ariño, Marta Consuegra-Fernández, Noelia Armiger-Borràs, Belchin Kostov, Manuel Ramos-Casals, Pilar Brito-Zerón and Francisco Lozano

1st scientific article

IF: 5.16 (Q2)

How hepatitis C virus modifies the immunological profile of Sjögren syndrome: analysis of 783 patients

Pilar Brito-Zerón*, **Hoda Gheitasi***, Soledad Retamozo1, Albert Bové1, María Londoño3, Jose-Maria Sánchez-Tapias3, Miguel Caballero4, Belchin Kostov5, Xavier Forns3, Srini V. Kaveri6 and Manuel Ramos-Casals1,2*

In this first scientific article, which analyzes how hepatitis C virus modifies the immunological profile of Sjögren syndrome in a large cohort of patient, PhD student Hoda Gheitasi contributed as 1st co-uthor by performing the following tasks:

- Analysis and interpretation of data.

- Acquisition of data

- Drafting the article

- Revising it critically for important intellectual content.

*Both co-authors contributed equally to this work.

The work was performed at the Sjögren Syndrome Research Group, Laboratory of Autoimmune Diseases Josep Font, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain. Finally, this scientific article has not been used for the realization of any other doctoral thesis, except the current one.

2nd scientific article

Etiopathogenic Role of Surfactant Protein D in the Clinical and Immunological Expression of Primary Sjögren Syndrome

María José Soto-Cárdenas, Myriam Gandía, Pilar Brito-Zerón, Maria Teresa Arias, Noelia Armiger, Albert Bové, Xavier Bosch, Soledad Retamozo, Miriam Akasbi, Marta Pérez-De-Lis, **Hoda Gheitasi**, Belchin Kostov, Roberto Pérez-Alvarez, Antoni Siso-Almirall, Francisco Lozano, and Manuel Ramos-Casals

In this second scientific article, in which the etiopathogenic role of surfactant protein D is evaluated in the clinical and immunological expression of Primary Sjögren Syndrome, PhD student Hoda Gheitasi contributed as the 11th author by performing the following tasks:

- Perform part of the genetic polymorphisms

- Revising it critically for important intellectual content

- Final approval of the published version

The work was performed at the Sjögren Syndrome Research Group, Laboratory of Autoimmune Diseases Josep Font, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain.

3rd scientific article

Gene Variation at Immunomodulatory and Cell Adhesion Molecules Loci Impacts Primary Sjögren's Syndrome

Sergi Casadó-Llombart 1†, **Hoda Gheitasi 2†**, Silvia Ariño1, Marta Consuegra-Fernández 1, Noelia Armiger-Borràs 1, Belchin Kostov 3,4,5, Manuel Ramos-Casals 2, Pilar Brito-Zerón6,7* and Francisco Lozano1,8,9*

In this last 3th scientific article, in which it was evaluated how gene variation at immunomodulatory and cell adhesion molecules loci impacts Sjögren's Syndrome, PhD student Hoda Gheitasi contributed as first co-author by performing the following tasks:

- Collection of clinical information and blood samples
- Performing genetic studies
- Analysis and interpretation of data
- Drafting the article
- Revising it critically for important intellectual content
- Final approval of the submitted version

These tasks were carried out equally by both co-authors of the work.

The work was performed at the Sjögren Syndrome Research Group, Laboratory of Autoimmune Diseases Josep Font and the Immunoreceptors del Sistema Innat i Adaptatiu, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain. Finally, this scientific article has not been used for the realization of any other doctoral thesis, except the current one.

SCIENTIFIC ARTICLES

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RESEARCH ARTICLE

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How hepatitis C virus modifies the immunological profile of Sjögren syndrome: analysis of 783 patients

Pilar Brito-Zerón[1,2†], Hoda Gheitasi[1†], Soledad Retamozo[1], Albert Bové[1], María Londoño[3], Jose-Maria Sánchez-Tapias[3], Miguel Caballero[4], Belchin Kostov[5], Xavier Forns[3], Srini V. Kaveri[6] and Manuel Ramos-Casals[1,2*]

Abstract

Introduction: We conducted a study to analyze how infection by hepatitis C virus (HCV) may influence the immunological serum pattern of patients with Sjögren syndrome (SS).

Methods: Since 1994, we have tested serum HCV-IgG antibodies in 783 patients with SS diagnosed according to the 1993 European classification criteria. The immunological profile at diagnosis was compared according to the presence or absence of HCV.

Results: Of the 783 patients with SS, 105 (13.4 %) tested positive for HCV-IgG antibodies (88 females, 17 males, mean age at SS diagnosis: 62.9 years). Multivariate analysis showed that patients with SS-HCV had a higher mean age and a higher frequency of low C3/C4 levels, cryoglobulins, and hematological neoplasia compared with patients without HCV. The frequency of anti-La antibodies compared with anti-Ro antibodies was higher in patients with SS-HCV (17 % vs. 15 %) and lower in patients without HCV infection (30 % vs. 43 %). The frequency of concomitant detection of the three main cryoglobulin-related markers (cryoglobulins, rheumatoid factor activity, and C4 consumption) was threefold higher in patients with SS-HCV compared with patients without HCV. SS-HCV patients with genotype 1b showed the highest frequencies of immunological abnormalities related to cryoglobulins and the lowest frequencies of anti-Ro/La antibodies.

Conclusions: We found HCV infection in 13 % of a large series of Spanish patients with SS. The HCV-driven autoimmune response was characterized by a lower frequency of anti-Ro/La antibodies, an abnormal predominance of anti-La among anti-Ro antibodies, and a higher frequency of cryoglobulinemic-related immunological markers in comparison with patients without HCV infection. This immunological pattern may contribute to the poor outcomes found in patients with SS-HCV.

Introduction

Sjögren syndrome (SS) is a systemic autoimmune disease that mainly affects the exocrine glands. This leads to dryness of the main mucosal surfaces, such as the mouth, eyes, nose, pharynx, larynx, and vagina [1]. The disease overwhelmingly affects middle-aged women but may also affect children, men, and older patients. The clinical spectrum of SS extends from dryness affecting the main mucosal surfaces to systemic involvement (extraglandular manifestations). SS may be a serious disease with excess mortality, which is related mainly to systemic involvement and hematological cancer [2].

In the etiopathogenesis of SS, a specific combination of individual genetic predisposition (intrinsic factors) and environmental agents (extrinsic factors) may be central to the development of the disease [3]. Viruses have always been considered one of the main exogenous culprits implicated in the etiopathogenesis of SS, and the hepatitis C virus (HCV) is a principal candidate [4]. In the last 15 years, several experimental, virological, and clinical studies have shown a close association between

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HCV and SS [5], suggesting that there may be shared immune-mediated etiopathogenic mechanisms. It sounds reasonable to investigate the role of the human ribonucleoproteins among these mechanisms. Human La protein is an essential factor in the biology of both coding and non-coding RNAs, is one of the principal autoantigens implicated in the etiopathogenesis of SS, and has been shown to play a key role in the initiation of HCV translation [6]. It could be hypothesized that patients carrying antibodies against Ro/La ribonucleoproteins are protected against chronic HCV infection.

The study of a large cohort of SS patients who were tested for HCV infection may help characterize the immunological profile of SS according to the presence or absence of HCV and reveal possible relationships between the main virological HCV features and the immunological expression of a systemic autoimmune disease characterized by an autoimmune response against human ribonucleoproteins, some of which have also been implicated in the translation and replication of HCV.

Methods

Since 1994, we have tested 783 consecutive patients with primary SS diagnosed according to the 1993 European classification criteria for serum HCV-IgG antibodies [7]; patients with concomitant systemic autoimmune diseases other than SS were excluded. Fulfillment of the 2002 American-European criteria [8] and the preliminary 2012 American College of Radiology (ACR) criteria [9] was retrospectively evaluated: 470 (60 %) fulfilled the 2002 criteria, and 499 (64 %) the preliminary 2012 criteria (29 patients fulfilled the 2012 criteria but not the 2002 criteria since they had positive rheumatoid factor (RF) and antinuclear antibodies (ANA) titers above 320, but with negative Ro/La antibodies/salivary gland biopsy).

HCV infection was defined as a positive serological result for serum HCV antibodies in at least two determinations. Anti-HCV antibodies were detected by second-generation enzyme-linked immunosorbent assay (ELISA) between 1994 and 1998 and third-generation ELISA since 1998; all patients who tested positive for the second-generation ELISA were re-tested with the third-generation test. Serum HCV-RNA was detected by polymerase chain reaction, viral load by real-time polymerase chain reaction (COBAS TaqMan HCV Test, Roche Diagnostics, Manheim, Germany), and HCV genotype by restriction fragment-length polymorphism of the 5′ non-coding region of the HCV genome, as previously described [10]. Virological studies (serum HCV-RNA detection, viral load, and genotype) were carried out according to clinical reasons and were not available in all patients. SS patients who tested HCV antibody-positive but HCV-RNA-negative were considered as having a past HCV infection that was resolved, whereas those

who tested positive for serum HCV-RNA were considered currently chronic HCV-infected.

A protocol form was used to retrospectively record the main characteristics of patients, including sex, age at diagnosis of SS (defined as the age when the patient fulfilled the current classification criteria), diagnostic tests for SS (ocular tests, salivary scintigraphy, salivary gland biopsy defined according to the recommendations of the European Community Study Group) [7], virological features (serum HCV-IgG, serum HCV-RNA, maximum viral load in the absence of anti-HCV therapies, HCV genotype), and adverse outcomes (neoplasia, death) until the last visit or death. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona (Spain), and the study design conformed to current Spanish ethical standards. Owing to the retrospective, observational, and anonymous nature of the study, informed patient consent was not required.

Immunological studies

Immunological tests were made by using commercial techniques standardly used in the Spanish public healthcare system, including ANA (indirect immunofluorescence using mouse liver and Hep-2 cells as substrate), precipitating antibodies to extractable nuclear antigens (Ro/SS-A, La/SS-B, U1-snRNP, and Sm; enzyme-linked immunoassay), and RF (nephelometry). Complement was measured by determination of C3 and C4 levels by nephelometry (BNII nephelometer; Dade Behring, Manburg, Germany). Serum cryoglobulins were measured after centrifugation. Blood samples were obtained and maintained at 37 °C for 30 min before separation. Serum was prepared by centrifuging at 37 °C for 10 min at 2500 revolutions per minute. Fresh centrifuged serum was incubated at 4 °C for 7 days after collection and examined for cryoprecipitation. Cryoglobulins were further analyzed by immunofixation when more than 5 % of cryoprecipitate was available. Serum monoclonal immunoglobulins were analyzed by immunofixation electrophoresis on agarose gels with specific antisera to IgG, IgM, IgA, and κ and λ chains at diagnosis and every year during the follow-up. Immunofixation was performed by using a Helena Immunofixation Agarose Kit (Helena Laboratories, Beaumont, TX, USA) in accordance with the instructions of the manufacturer.

Statistical analysis

Descriptive data are presented as mean and standard deviation for continuous variables and as number and percentage for categorical variables. Qualitative differences were analyzed by using the chi-squared and Fisher's exact tests. When several independent variables appeared to be statistically significant in the univariate analysis, logistic regression was made in a multivariate analysis to rule out

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possible confounding variables. To compare quantitative parameters, the Student's *t* test was used in large samples of similar variance, and the non-parametric Mann–Whitney *U* test was used for small samples. A *P* value of less than 0.05 indicated statistical significance. The analysis was carried out by using the 18.0 SPSS program (SPSS, Chicago, IL, USA).

Results

Prevalence of HCV infection

Of the 783 patients with SS, 105 (13.4 %) tested positive for HCV-IgG antibodies (88 females and 17 males, with a mean age at SS diagnosis of 62.9 years). The prevalence

varied according to the SS criteria fulfilled: 8 % (38/470) in patients fulfilling the 2002 criteria, 10 % (51/499) in patients fulfilling the 2012 ACR criteria, and 19 % (54/284) in patients fulfilling only the 1993 criteria; the highest percentage was found in the 29 patients who fulfilled the 2012 criteria but not the 2002 criteria (13 patients were HCV-positive, 45 %).

Comparison between HCV-IgG-positive and -negative patients

Table 1 summarizes the main features of patients according to the presence or absence of serum HCV-IgG antibodies. In the univariate analysis, patients with SS-HCV

Table 1 Main SS-related features of patients according to the presence or absence of serum HCV-IgG antibodies

	Negative HCV-IgG N = 678	Positive HCV-IgG N = 105	Bilateral *P* value
Mean age, years	56.36 ± 14.83	62.93 ± 11.86	<0.001*
Sex, male	45 (7 %)	17 (16 %)	**0.003**
Dry mouth	661 (98 %)	103 (98 %)	1
Dry eye	661 (98 %)	104 (99 %)	0.493
Altered ocular tests	559/609 (92 %)	82/88 (93 %)	0.834
Altered parotid scintigraphy	486/554 (88 %)	45/54 (83 %)	0.389
Positive salivary gland biopsy	198/309 (64 %)	21/30 (70 %)	0.556
Criteria SS			
- 1993 only	230 (34 %)	54 (51 %)	**<0.001**
- 2002	432 (64 %)	38 (26 %)	
- ACR only	16 (2 %)	13 (12 %)	
Antinuclear antibody[+]	568/676 (84 %)	79/104 (76 %)	**0.05**
Rheumatoid factor[+]	267/663 (40 %)	57/102 (56 %)	**0.004**
Anti-Ro/SS-A[+]	292/676 (43 %)	16/103 (15 %)	<0.001*
Anti-La/SS-B[+]	200/676 (30 %)	17/103 (17 %)	**0.006**
Monoclonal gammopathy	85/492 (17 %)	35/75 (47 %)	**<0.001**
Type of monoclonal band			
- mIgA	9 (11 %)	1 (3 %)	**0.047**
- mIgG	47 (55 %)	13 (37 %)	
- mIgM	18 (21 %)	15 (43 %)	
- Free chains	11 (13 %)	6 (17 %)	
Type of monoclonal light chain			
- Kappa:lambda	50:35	19:16	0.65
Cryoglobulin[+]	41/626 (7 %)	63/104 (61 %)	<0.001*
Low C3 levels, <0.82 g/l	70/660 (11 %)	37/103 (36 %)	<0.001*
Low C4 levels, <0.11 g/l	45/660 (7 %)	49/103 (48 %)	<0.001*
Hematological neoplasia	26 (4 %)	9 (9 %)	0.04*
Neoplasia	54 (8 %)	21 (20 %)	<0.001*
Death	52 (8 %)	35 (33 %)	**<0.001**

*Statistically significant in the multivariate model.
SS Sjögren syndrome, *HCV* hepatitis C virus, *IgG* immunoglobulin G, *ACR* American College of Rheumatology, *SS-A* Sjögren syndrome A antigen, *SS-B* Sjögren syndrome B antigen, *mIg* circulating monoclonal immunoglobulin, *C3* complement component 3, *C4* complement component 4Bold numbers: statistically-significant differences in the univariate analysis (p<0.05)

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Table 2 Main SS-related features of patients according to the HCV genotypes (1a, 1b, and non-1 genotypes)

	Genotype 1a N = 8	Genotype 1b N = 30	Non-1 genotypes N = 5	Bilateral P value
Mean age, years	62.62 ± 12.62	64.93 ± 9.81	46.00 ± 8.15	**0.002**
Sex, male	0 (0 %)	8 (27 %)	2 (40 %)	0.182
Dry mouth	8 (100 %)	30 (100 %)	5 (100 %)	1.000
Dry eye	8 (100 %)	30 (100 %)	5 (100 %)	1.000
Altered ocular tests	5/6 (83 %)	23/24 (96 %)	4/5 (80 %)	0.381
Altered parotid scintigraphy	4/5 (80 %)	14/17 (82 %)	2/3 (67 %)	0.822
Positive salivary gland biopsy	5/5 (100 %)	3/5 (60 %)	0/2 (0 %)	**0.037**
Criteria SS				
- 1993 only	3 (37 %)	20 (67 %)	2 (40 %)	0.175
- 2002	5 (63 %)	7 (23 %)	3 (60 %)	
- ACR only	0 (0 %)	3 (10 %)	0 (0 %)	
Antinuclear antibody[+]	6 (75 %)	26 (87 %)	4 (80 %)	0.709
Rheumatoid factor[+]	3 (37 %)	16/29 (55 %)	1 (20 %)	0.284
Anti-Ro/SS-A[+]	2 (25 %)	3 (10 %)	2 (40 %)	0.185
Anti-La/SS-B[+]	1 (12 %)	5 (17 %)	2 (40 %)	0.410
Monoclonal gammopathy	3 (37 %)	13/26 (50 %)	0/1 (0 %)	0.535
Type of monoclonal band				
- mIgA/mIgG/mIgM/free chains	0/0/2/1	1/6/5/1	0/0/0/0	0.34
- Kappa:lambda light chains	1:2	6:7	0	1.000
Cryoglobulin[+]	4 (50 %)	20/29 (69 %)	0 (0 %)	**0.014**
Low C3 levels, <0.82 g/l	3 (37 %)	12 (40 %)	3 (60 %)	0.677
Low C4 levels, <0.11 g/l	4 (50 %)	13 (43 %)	2 (40 %)	0.926
Hematological neoplasia	1 (12 %)	3 (10 %)	1 (20 %)	0.809
Neoplasia	1 (12 %)	6 (20 %)	1 (20 %)	0.886
Death	4 (50 %)	9 (30 %)	0 (0 %)	0.161
Viral load > 5,000,000	2 (25 %)	11/28 (39 %)	0/5 (0 %)	0.095
Max viral load (log)	6.36 ± 0.39	6.13 ± 0.55	5.96 ± 0.38	0.350

SS Sjögren syndrome, *HCV* hepatitis C virus, *IgG* immunoglobulin G, *ACR* American College of Radiology, *SS-A* Sjögren syndrome A antigen, *SS-B* Sjögren syndrome B antigen, *mIg* circulating monoclonal immunoglobulin, *C3* complement component 3, *C4* complement component 4Bold numbers: statistically-significant differences in the univariate analysis (p<0.05)

We found HCV infection in 13 % of a large series of Spanish patients who fulfilled the 1993 classification criteria for SS (8 % in patients fulfilling the more restrictive 2002 criteria). Various studies have analyzed the prevalence of chronic HCV infection in patients with primary SS, and most have found a higher prevalence than in the general population, although the results vary according to the geographic area. Studies from southern Europe describe a prevalence ranging from 10 % to 20 % (14 % using ELISA-3 and 5 % to 19 % using RIBA-2) [5]. In contrast, studies from Scandinavia and the USA have found no association between SS and HCV (prevalence of less than 1 %) and this is possibly due to the lower prevalence of HCV infection in these countries compared with the Mediterranean area [17]. We tested the largest SS population from a single center for HCV infection and found a prevalence 10-fold higher than that found in the general Spanish population [18], although it is possible that there may be a potential referral bias.

Demographically, SS-HCV was characterized by a comparatively reduced female-to-male ratio (5:1 vs. 14:1 in patients with primary SS) and an older age at SS diagnosis. The clinical phenotype of SS-HCV was indistinguishable from that of primary SS, and there were no significant differences in the prevalence of sicca features and the corresponding diagnostic tests. The sialotropism of HCV [4] may explain the close association with SS, including the results of salivary gland biopsies in patients with HCV. Experimental studies have reported that the envelope proteins of HCV may recruit lymphocytes in the salivary glands, leading to the formation of lymphocytic infiltrates, as occurs in primary SS (focal sialadenitis) [13–15]. De Vita et al. [19] first detected HCV in human salivary glands,

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Table 3 Combination of the main SS-related immunological profiles (ANA/RF, Ro/La, and cryoglobulinemic-related markers) according to the presence or absence of serum HCV-IgG antibodies

ANA/RF combination	Negative HCV-IgG	Positive HCV-IgG	Bilateral *P* value
	N = 306	N = 74	
ANA and RF	94 (31 %)	29 (39 %)	<0.001
Isolated ANA	200 (65 %)	32 (43 %)	
Isolated RF	12 (4 %)	13 (18 %)	
Ro/La combination	Negative HCV-IgG	Positive HCV-IgG	Bilateral *P* value
	N = 314	N = 21	
Anti-Ro and anti-La antibodies	178 (57 %)	12 (57 %)	0.014
Isolated anti-Ro antibodies	114 (36 %)	4 (19 %)	
Isolated anti-La antibodies	22 (7 %)	5 (24 %)	
Cryoglobulinemic-related markers combination	Negative HCV-IgG	Positive HCV-IgG	Bilateral *P* value
	N = 38	N = 60	
Cryoglobulins+RF+low C4	7 (18 %)	28 (47 %)	0.019
Cryoglobulins+RF	12 (32 %)	13 (22 %)	
Cryoglobulins+low C4	6 (16 %)	10 (17 %)	
Isolated cryoglobulins	13 (34 %)	9 (15 %)	

SS Sjögren syndrome, *ANA* antinuclear antibodies, *RF* rheumatoid factor, *HCV* hepatitis C virus, *IgG* immunoglobulin G, *C4* complement component 4

and two additional studies [20, 21] have demonstrated the capability of the HCV to infect and replicate in the salivary gland tissue of HCV patients with sicca syndrome/SS. The reasons for the specific predilection of HCV for infecting exocrine gland tissue are unknown.

The autoantibody profile of patients with HCV-related SS is characterized by a higher frequency of RF and a lower frequency of Ro/La antibodies [5]. This immunological pattern influences the fulfillment of the new classification criteria proposed after the 1993 criteria, making fulfillment of the 2002 criteria (positivity for Ro/La is mandatory in the absence of a positive salivary gland result) more difficult than fulfillment of the 2012 criteria (these criteria allow the inclusion of patients with positive ANA/RF even in the absence of Ro/La antibodies). As the results of this study show, the prevalence of HCV infection in patients with SS may vary widely according to the set of criteria used to classify patients with SS.

In patients with SS, the HCV-driven autoimmune response is dominated principally by the presence of mixed cryoglobulins, reported in nearly two thirds of patients with SS-HCV (a ninefold higher prevalence with respect to patients with primary SS). Cryoglobulins play a predominant role in the global immunological pattern of these patients and is closely associated with positive RF, monoclonal gammopathy, and low C4 levels, whose frequencies (either isolated or in combination) were higher than those observed in patients without HCV. In addition, we found significant differences in the serum monoclonal expression (frequency and heterogeneity) of patients with SS according to the presence or absence of

HCV infection. The prevalence of circulating mIgs in patients with SS-HCV was threefold higher than in patients without HCV, and mIgMκ, which was closely related to mixed cryoglobulinemia, was the most common type of circulating mIg, whereas in patients without HCV, mIgGk was the predominant circulating monoclonal band. We also found that SS-HCV patients with monoclonal gammopathy had a more restrictive monoclonal expression (overwhelmingly limited to either mIgMk or mIgG) compared with patients without HCV, who presented all types of monoclonal heavy and light chains. This suggests that HCV may play an important role in the clonal selection of specific B cells [22].

The lymphotropism of HCV links the virus not only to the synthesis of cryoglobulins but also to the development of lymphoma [23], and we found a higher frequency of hematological neoplasia in patients with SS-HCV compared with those without HCV. Lymphomagenesis in patients with HCV might be initiated by chronic stimulation of polyclonal B cells by the virus [24] and the compartmentalization of HCV quasispecies in blood mononuclear cells [25], with the posterior development of specific B-cell clonal expansions and pro-carcinogenic mutations [26, 27], which are similar to the etiopathogenic mechanisms of lymphoma development reported in primary SS [24, 28, 29]. Primary SS is the systemic autoimmune disease with the highest risk of lymphoma development [30]: we found that the combination of HCV and SS doubled the risk reported in patients with SS alone. Both SS and chronic HCV infection are characterized by underlying B-cell hyperactivity which predisposes

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Table 4 Main SS-related features of the 17 SS-HCV patients with anti-La antibodies in comparison with the 200 SS patients with anti-La antibodies without HCV infection

	Anti-La⁺ SS patients with HCV infection N = 17	Anti-La⁺ SS patients with no HCV infection N = 200	Bilateral *P* value
Mean age, years	59.59 ± 11.87	51.04 ± 15.77	**0.03**
Sex, male	4 (23 %)	8 (4 %)	0.009*
Dry mouth	16 (94 %)	197 (99 %)	0.280
Dry eye	17 (100 %)	196 (98 %)	1.000
Altered ocular tests	15/16 (94 %)	176/187 (94 %)	1.000
Altered parotid scintigraphy	9/10 (90 %)	151/165 (91 %)	0.602
Positive salivary gland biopsy	3/3 (100 %)	55/61 (90 %)	1.000
Antinuclear antibody⁺	14 (82 %)	170/199 (85 %)	0.723
Rheumatoid factor⁺	13/17 (77 %)	112/191 (59 %)	0.199
Anti-Ro/SS-A⁺	12 (71 %)	178 (89 %)	**0.044**
Monoclonal gammopathy	7/13 (54 %)	33/166 (20 %)	**0.01**
Type of monoclonal band			
- mIgA	0 (0 %)	3 (9 %)	0.87
- mIgG	4 (57 %)	17 (52 %)	
- mIgM	2 (29 %)	9 (27 %)	
- Free chains	1 (14 %)	4 (12 %)	
Type of monoclonal light chain			
- Kappa:lambda	2:5	23:10	**0.041**
Cryoglobulin⁺	9 (53 %)	19/188 (10 %)	**<0.001**
Low C3 levels, <0.82 g/l	9 (53 %)	22/196 (11 %)	**<0.001***
Low C4 levels, <0.11 g/l	11 (65 %)	14/196 (7 %)	**<0.001***
Hematological neoplasia	0 (0 %)	7 (3.5 %)	1.000
Neoplasia	4 (23.5 %)	17 (8.5 %)	0.067
Death	7 (41 %)	16 (8 %)	**0.001**

*Statistically significant in the multivariate model

SS Sjögren syndrome, *HCV* hepatitis C virus, *SS-A* Sjögren syndrome A antigen, *mIg* circulating monoclonal immunoglobulin, *C3* complement component 3, *C4* complement component 4Bold numbers: statistically-significant differences in the univariate analysis (p<0.05)

to monoclonal B-cell selection [31]; therefore, SS-HCV may present one of the highest risks of overt B-cell lymphoma of all systemic autoimmune diseases.

Human La protein is known to be an essential host factor for the translation and replication of HCV RNA [32]. Translation of HCV is an essential step of viral replication and is mediated by an internal ribosome entry site, and the ribonucleoprotein La is a potent regulator for the enhancement of HCV replication [33]. We tested the hypothesis that patients carrying anti-La antibodies could be protected against chronic HCV infection. Unfortunately, the results suggest that serum anti-La antibodies do not play a significant protective role against chronification of HCV infection in patients with SS. None of the seven SS patients who had a resolved HCV infection (positive HCV-IgG with negative HCV-RNA) carried anti-La antibodies. In addition, we found no significant differences in the epidemiological, clinical, and

immunological profile of La-positive patients according to the presence or absence of HCV infection.

We found an abnormal Ro/La immunological pattern according to the presence or absence of HCV infection. First, we found that the prevalence of anti-Ro/La antibodies was significantly reduced in patients with SS-HCV compared with patients without HCV. Second, we found more patients with SS-HCV were carrying La autoantibodies than those carrying anti-Ro autoantibodies, and this is in clear opposition to results found in SS patients without HCV. Third, we found that nearly one quarter of Ro/La⁺ SS-HCV patients carried isolated anti-La antibodies, an immunological pattern rarely reported in primary SS (<5 %). These findings suggest that, in patients with SS, the autoimmune response against the human La protein is significantly altered by the concomitant presence of HCV infection, an abnormal response probably related to the use of human ribonucleoproteins by the virus.

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The study has some limitations related to the small number of patients included in some comparisons, such as the low number of patients with resolved HCV infection (only 7) or the low percentage of HCV patients with available viral genotyping in whom a salivary gland biopsy was carried out (only 12 out of 43 patients). In these comparisons, the statistically significant results should be taken with caution and require confirmation by studies including a large number of patients.

Conclusions

In summary, we found HCV infection in 13 % of a large series of Spanish patients with SS. The HCV-driven autoimmune response in patients with SS-HCV is characterized by a lower frequency of autoantibodies against Ro and La human ribonucleoproteins, an abnormal predominant presence of anti-La among anti-Ro antibodies, and a higher frequency of cryoglobulinemic-related immunological markers (including positive RF) in comparison with SS patients without HCV. This immunological pattern influences the fulfillment of the SS classification criteria and may be related to the increased prevalence of hematological neoplasia that we found in patients with SS-HCV.

Abbreviations

ACR: American College of Rheumatology; ANA: Antinuclear antibodies; C3: Complement component 3; C4: Complement component 4; ELISA: Enzyme-linked immunosorbent assay; HCV: Hepatitis C virus; HEp-2: Human epithelial type 2; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IgM: Immunoglobulin M; mIg: Circulating monoclonal immunoglobulin; RF: Rheumatoid factor; Sm: Smith; SS: Sjögren syndrome; SS-A: Sjögren syndrome A antigen; SS-B: Sjögren syndrome B antigen; U1-snRNP: U1 small nuclear ribonucleoprotein particle.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PB-Z participated in conception and design and in analysis and interpretation of data. SVK participated in conception and design. MR-C participated in conception and design, in analysis and interpretation of data, and in statistical analysis. HG and XF participated in analysis and interpretation of data. BK participated in analysis and interpretation of data and in statistical analysis. All authors participated in acquisition of data and in drafting the article or revising it critically for important intellectual content. They agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. They have all read and approved the final manuscript.

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Etiopathogenic Role of Surfactant Protein D in the Clinical and Immunological Expression of Primary Sjögren Syndrome

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ABSTRACT. Objective. To analyze the etiopathogenic role of genetic polymorphisms and serum levels of surfactant protein-D (SP-D) in primary Sjögren syndrome (pSS).

Methods. We analyzed 210 consecutive patients with pSS. *SFTPD* genotyping (M11T polymorphism rs721917) was analyzed by sequence-based typing and serum SP-D by ELISA.

Results. Thirty-two patients (15%) had the Thr11/Thr11 genotype, 80 (38%) the Met11/Met11 genotype, and 96 (46%) the Met11/Thr11 genotype; 2 patients could not be genotyped. Patients carrying the Thr11/Thr11 genotype had a higher prevalence of renal involvement (13% vs 1% and 4% in comparison with patients carrying the other genotypes, p = 0.014). Serum SP-D levels were analyzed in 119 patients (mean 733.94 ± 49.88 ng/ml). No significant association was found between serum SP-D levels and the SP-D genotypes. Higher mean values of serum SP-D were observed in patients with severe scintigraphic involvement (851.10 ± 685.69 vs 636.07 ± 315.93 ng/ml, p = 0.038), interstitial pulmonary disease (1053.60 ± 852.03 vs 700.36 ± 479.33 ng/ml, p = 0.029), renal involvement (1880.64 ± 1842.79 vs 716.42 ± 488.01 ng/ml, p = 0.002), leukopenia (899.83 ± 661.71 vs 673.13 ± 465.88 ng/ml, p = 0.038), positive anti-Ro/SS-A (927.26 ± 731.29 vs 642.75 ± 377.23 ng/ml, p = 0.006), and positive anti-La/SS-B (933.28 ± 689.63 vs 650.41 ± 428.14 ng/ml, p = 0.007), while lower mean values of serum SP-D were observed in patients with bronchiectasis (489.49 vs 788.81 ng/ml, p = 0.019).

Conclusion. In pSS, high SP-D levels were found in patients with severe glandular involvement, hypergammaglobulinemia, leukopenia, extraglandular manifestations, and positive anti-Ro/La antibodies. The specific association between SP-D levels and pulmonary and renal involvements may have pathophysiological implications. (First Release Nov 1 2014; J Rheumatol 2015;42:111–18; doi:10.3899/jrheum.140394)

Key Indexing Terms:
SJÖGREN SYNDROME   SURFACTANT   INNATE IMMUNITY

Sjögren syndrome (SS) is a systemic autoimmune disease that presents with sicca symptomatology of the main mucosal surfaces[1]. The main sicca features (xerophthalmia and xerostomia) are determined by specific ocular (Rose Bengal staining, Schirmer test) and oral (salivary flow measurement, parotid scintigraphy) tests. The histological

hallmark is focal lymphocytic infiltration of the exocrine glands, determined by biopsy of the minor labial salivary glands[2]. The spectrum of the disease extends from sicca syndrome to systemic involvement (extraglandular manifestations) and may be complicated by the development of lymphoma. Patients with SS present a broad spectrum of analytical features (cytopenias, hypergammaglobulinemia) and autoantibodies, of which antinuclear antibodies are the most frequently detected, anti-Ro/SSA the most specific, and cryoglobulins and hypocomplementemia the main prognostic markers[3].

The study of innate immunity has led to an increasing interest in autoimmune diseases. Innate immunity is an ancient and universal form of host defense against invading pathogens, but also an integral part of a broader system responsible for the homeostasis of the internal environment in multicellular organisms. Its main function is to detect non-self or modified-self molecules and eliminate them, whether directly or through the stimulation of an adaptive immune response. To do this, the innate immune system relies on a relatively small number of nonpolymorphic and broadly distributed receptors that have evolved to mainly recognize the so-called pathogen-associated molecular patterns, which are conserved pathogenic structures, essential for pathogen survival, and not shared by the host[4].

Collectins are pattern-recognition receptors that preferentially bind to carbohydrate moieties expressed on a variety of pathogens, thereby enhancing aggregation, opsonization, or complement activation[5]. Surfactant protein-D (SP-D), a member of the collectin family, is a highly versatile innate immune molecule involved in a range of immune functions, including the neutralization and clearance of microorganisms and downregulation of allergic/inflammatory processes. Its receptor is located in a wide variety of cells, including endothelial cells, B lymphocytes, and antigen-presenting cells. In addition, the SP-D receptor can bind to the major IgG subclasses, suggesting it may play a role as a link between the innate and adaptive immune system[6]. SP-D is primarily synthesized by the respiratory epithelium (alveolar type II cells)[7], but is also expressed by extrapulmonary epithelia[8,9,10]. Serum levels of SP-D are genetically regulated, and a common polymorphism in the SP-D gene on chromosome 10 (Met11Thr) has been associated with serum levels and multimerization of SP-D[11,12]. The Thr11 variant has been associated with reduced oligomerization, reduced binding capacity of microbes, and low serum levels in healthy subjects[12].

The recent detection of SP-D in epithelial cells and in luminar material from the ducts of glandular tissues, including lacrimal and salivary glands[10], might suggest an etiopathogenic role in primary SS (pSS) which, pathogenically, is defined as an "autoimmune epithelitis"[13]. Our study investigated the relationship between genetic polymorphisms and serum levels of SP-D on the one hand, and clinical and immunological disease expression on the other hand, in a large series of patients with pSS.

MATERIALS AND METHODS

We analyzed 210 consecutive patients who fulfilled the current classification criteria for pSS[14,15]. In all patients, an exhaustive evaluation was made, discarding other causes of sicca syndrome (coexisting systemic autoimmune diseases, chronic viral infections, metabolic disorders, and preexisting lymphoma). Extraglandular involvement was evaluated according to the 2010 European League Against Rheumatism (EULAR) SS disease activity index at the time of blood extraction[16]; pulmonary involvement was defined by the presence of respiratory symptoms associated with altered pulmonary diagnostic tests (pulmonary function tests and/or computed tomography), although the EULAR Sjögren Syndrome Disease Activity Index (ESSDAI) definition also included asymptomatic patients with altered pulmonary imaging and those with persistent respiratory symptoms but normal imaging studies[16]. With respect to renal involvement, the ESSDAI includes both tubular and glomerular involvements[16]. Salivary scintigraphic results were classified according to the criteria proposed by Schall and Di Chiro[17]. Samples of blood donors ethnically matched were used for *SFTPD* genotyping in healthy controls. Clinical and laboratory data were collected and computerized according to our standard department protocol[18]. The study was approved by the Ethics Committee of the Hospital Clinic (Barcelona, Spain) and all patients gave informed, written consent. After blood extraction, all patients were followed up with regular visits at 6–12-month intervals, and the main outcomes (cardiovascular disease, infections, neoplasia, and death) were evaluated at the last visit.

SFTPD genotyping. Genomic DNA was extracted from EDTA-treated whole blood samples using the QIAamp DNA blood mini kit following the manufacturer's instructions (QIAGEN GmbH) and stored at –20°C until used. The *SFTPD* rs721917 polymorphism was genotyped using a typing technique based on PCR sequencing. In brief, a 317-bp fragment corresponding to exon 1 was obtained by PCR amplification using the sense 5'-AGC CCT AAA CCA TGT CCA TGA-3' and antisense 5'-AGG AAT GGT CAT TGG AAC TGT-3' primers and GoTaq DNA Polymerase (Promega). The cycling conditions were 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s; and finally, 1 cycle of 72°C for 7 min. Five ml of each PCR were treated with ExoSAP-IT (USB Corp.) and subjected to direct sequencing with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and the sense and antisense gene-specific primers mentioned above following the manufacturer's instructions. Sequencing reactions were analyzed on an automated capillary DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems).

Determination of serum SP-D levels. Serum samples were available from 119 patients. SP-D was measured by sandwich ELISA using the Human SP-D ELISA kit (Hycult Biotech, Cat No Hk335) following the manufacturer's instructions. All analyses were done in duplicate. The measurement range was between 6.3 and 400 ng/ml. According to the specifications included in the ELISA kit, although the standard measurement range is between 6.3 and 400 ng/ml, basal SP-D levels are highly variable and may range in healthy individuals from 200 ng/ml to 5200 ng/ml.

Statistical analysis. Descriptive data are presented as the mean and SD for continuous variables or number and percentage for categorical variables. Qualitative variables were compared using the chi-square test and Fisher's exact test. Quantitative variables were analyzed with the Student t test. All significance tests were 2-tailed and values of $p < 0.05$ were considered significant. Bonferroni correction was applied for the variables that were statistically significant in the univariate analysis. The statistical analysis was performed using the SPSS program (IBM SPSS Statistics, version 20).

RESULTS

Prevalence and clinical relevance of Met11Thr SP-D

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RESULTS

Prevalence and clinical relevance of Met11Thr SP-D

polymorphisms. Of the 210 patients with pSS, 96 (46%) carried the heterozygous Met11/Thr11 genotype, 80 (38%) the homozygous Met11/Met11 genotype, and 32 (16%) the homozygous Thr11/Thr11 genotype; DNA could not be genotyped in 2 patients. Genotype distribution was similar in healthy controls (42%, 40%, and 18% for the Met11/Thr11, Met11/Met11, and Thr11/Thr11 genotypes, respectively).

Analysis of the relationship between Met11Thr SP-D polymorphisms and the main epidemiological, clinical, and immunological characteristics of pSS (Table 1) showed statistically significant associations only for a higher prevalence of renal involvement in patients carrying the homozygous Thr11/Thr11 genotype (13% vs 1% and 4% in patients carrying the Met11/Thr11 and Met11/Met11 genotypes, respectively, p = 0.014). A significant trend was found in patients carrying the homozygous Thr11/Thr11 genotype for a lower frequency of vasculitis (0% vs 10% and 6%), and a higher frequency of severe parotid scintigraphy results (60% vs 48% and 46%), interstitial lung disease (ILD; 16% vs 8% and 8%), and positive anti-La antibodies (38% vs 21% and 30%) in comparison with patients carrying the other Met11Thr SP-D genotypes.

Serum SP-D levels and clinical/immunological expression. Serum SP-D levels were analyzed in 119 patients (mean 733.94 ± 49.88 ng/ml, median 588.72 ng/ml). No significant differences were found between serum SP-D levels and the SP-D genotypes (median serum levels of 577.59 ng/ml in Met11/Met11 genotype carriers, 633.49 ng/ml in Met11/Thr11 genotype carriers, and 519.62 ng/ml in Thr11/Thr11 genotype carriers, p = 0.53). Patients fulfilling the 2002 criteria[14] for SS showed median higher levels in comparison with those fulfilling the American College of Rheumatology criteria, although the differences were not significant (627.79 ± 70.69 vs 558.32 ± 60 ng/ml, p = 0.141).

Table 2 and Table 3 summarize the mean values of serum SP-D according to the presence or absence of the main epidemiological, clinical, and laboratory features at the time of blood extraction. Epidemiologically, no correlation was found between age and SP-D serum levels (Pearson correlation coefficient –0.082, bilateral p value = 0.377); males had higher mean values than females, although the difference was not significant (p = 0.192). Clinically, higher mean values of serum SP-D were observed in patients with severe involvement (grades III/IV) in parotid scintigraphy (851 ± 685.69 vs 636.07 ± 315.93 ng/ml, p = 0.038), renal

Table 1. Epidemiological, clinical, and immunological features of patients with pSS according to SP-D genotypes (Met11/Met11, Met11/Thr11, and Thr11/Thr11). Values are n (%) unless otherwise specified.

Characteristics	Met11/Met11, n = 80	Met11/Thr11, n = 96	Thr11/Thr11, n = 32	p
Male sex	5 (6)	7 (7)	1 (3)	0.837
Age, mean ± SD	54.56 ± 13.62	56.76 ± 13.99	60.28 ± 11.52	0.126
Dry mouth	79 (99)	93 (97)	32 (100)	0.46
Dry eyes	79 (99)	92 (96)	31 (97)	0.51
Altered ocular tests	70 (87)	89 (93)	32 (100)	0.47
Parotid scintigraphy grade III/IV	32/70 (46)	41/85 (48)	15/25 (60)	0.23
Positive salivary gland biopsy	27/39 (69)	33/47 (70)	12/14 (86)	0.46
Parotid enlargement	20 (25)	22 (23)	11 (34)	0.43
Fever	6 (8)	14 (15)	2 (6)	0.216
Arthralgia	49 (61)	50 (52)	21 (65)	0.29
Arthritis	8 (10)	12 (13)	8 (25)	0.22
Raynaud phenomenon	15 (18)	26 (27)	4 (13)	0.16
Vasculitis	5 (6)	10 (10)	0 (0)	0.13
Bronchiectasis	8 (10)	15 (16)	7 (22)	0.244
Pulmonary interstitial disease	6 (8)	8 (8)	5 (16)	0.374
Autoimmune liver disease	5 (6)	11 (11)	5 (16)	0.28
Renal involvement	3 (4)	1 (1)	4 (13)	0.014
Peripheral neuropathy	4 (5)	12 (13)	2 (6)	0.18
CNS involvement	5 (6)	11 (11)	3 (9)	0.49
ESR, mean ± SD	39.24 ± 30.53	35.77 ± 32.27	37.47 ± 29.54	0.504
Anemia, Hb < 10 g/l	13 (16)	15 (16)	4 (13)	0.880
Leukopenia, < 4 × 10⁹/l	16 (20)	25 (26)	5 (16)	0.397
Thrombocytopenia, < 100 × 10⁹/l	2 (2)	9 (9)	1 (3)	0.118
ANA+	64 (80)	86 (90)	28 (88)	0.186
RF+	35/79 (44)	36 (38)	13 (41)	0.660
Anti-Ro/SS-A+	28 (35)	30 (31)	12 (38)	0.769
Anti-La/SS-B+	24 (30)	20 (21)	12 (38)	0.134

pSS: primary Sjögren syndrome; SP-D: surfactant protein-D; CNS: central nervous system; ESR: erythrocyte sedimentation rate; ANA: antinuclear antibody; RF: rheumatoid factor.

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Table 2. Epidemiological and clinical features of patients with pSS according to SP-D serum levels.

Characteristics	SP-D levels, ng/ml, mean ± SD	Bilateral p
Sex		0.192
Male	992.07 ± 654.26	
Female	719.98 ± 525.30	
Xerostomia		0.665
Yes	739.42 ± 540.06	
No	603.45 ± 212.32	
Xerophthalmia		0.319
Yes	745.13 ± 540.75	
No	473.13 ± 146.83	
Ocular tests		0.477
Altered	751.05 ± 543.57	
Not altered	525.95 ± 119.06	
Parotid scintigraphy		0.038
Severe dysfunction grades III/IV	851.10 ± 685.69	
Grades I/II	636.07 ± 315.93	
Salivary biopsy		0.745
Positive	727.98 ± 526.35	
Negative	675.71 ± 565.14	
Parotid enlargement		0.113
Yes	863.93 ± 534.82	
No	688.92 ± 529.40	
Fever		0.943
Yes	745.33 ± 446.41	
No	734.64 ± 547.67	
Arthralgia		0.954
Yes	738.39 ± 567.01	
No	732.66 ± 491.20	
Arthritis		0.727
Yes	774.27 ± 557.82	
No	728.25 ± 532.02	
Raynaud phenomenon		0.430
Yes	649.48 ± 409.40	
No	753.46 ± 556.24	
Vasculitis		0.657
Yes	801.39 ± 447.94	
No	728.65 ± 544.45	
Pulmonary bronchiectasis		0.019
Yes	489.49 ± 219.74	
No	788.81 ± 566.88	
Pulmonary interstitial disease		0.029
Yes	1053.60 ± 852.03	
No	700.36 ± 479.33	
Renal involvement		0.002*
Yes	1880.64 ± 1842.79	
No	716.42 ± 488.01	
Peripheral neuropathy		0.629
Yes	672.51 ± 441.56	
No	745.38 ± 548.56	
CNS involvement		0.525
Yes	656.26 ± 358.89	
No	740.62 ± 572.02	

* Statistically significant in the Bonferroni correction. pSS: primary Sjögren syndrome; SP-D: surfactant protein-D; CNS: central nervous system.

Table 3. Laboratory and immunological features of patients with pSS according to the SP-D serum levels.

Characteristics	SP-D levels, ng/ml, mean ± SD	Bilateral p
Haemoglobin levels		0.736
< 10 g/l	769.97 ± 495.17	
> 10 g/l	727.84 ± 545.40	
White blood cell count		0.038
< 4 × 10^9/l	899.83 ± 661.71	
> 4 × 10^9/l	673.12 ± 465.88	
Platelet count		0.924
< 100 × 10^9/l	719.45 ± 553.04	
> 100 × 10^9/l	737.34 ± 535.32	
ANA		0.695
Positive, > 1/40	743.04 ± 559.24	
Negative	683.05 ± 292.86	
RF		0.064
Positive, > 25 UI/l	851.23 ± 677.74	
Negative	663.94 ± 411.43	
Anti-Ro/SS-A		0.006*
Positive	927.26 ± 731.29	
Negative	642.75 ± 377.23	
Anti-La/SS-B		0.007*
Positive	933.28 ± 689.63	
Negative	650.41 ± 428.14	
Monoclonal band		0.778
Positive	776.65 ± 401.17	
Negative	736.48 ± 607.55	
Cryoglobulins		0.563
Positive	817.60 ± 620.96	
Negative	724.02 ± 517.99	
C3 levels		0.442
< 0.82 g/l	828.77 ± 510.82	
> 0.82 g/l	720.52 ± 538.98	
C4 levels		0.246
< 0.11 g/l	536.75 ± 322.26	
> 0.11 g/l	752.29 ± 545.78	

* Statistically significant in the Bonferroni correction. pSS: primary Sjögren syndrome; SP-D: surfactant protein-D; ANA: antinuclear antibody; RF: rheumatoid factor; C3: complement factors.

interstitial involvement (1880.64 ± 1842.79 vs 716.42 ± 488.01 ng/ml, p = 0.002), and interstitial pulmonary disease (1053.60 ± 852.03 vs 700.36 ± 479.33 ng/ml, p = 0.029) while patients with pulmonary bronchiectasis had lower SP-D levels (489.49 ± 219.74 vs 789 ± 566.88 ng/ml, p = 0.019). With respect to laboratory variables, higher SP-D levels were found in patients with leukopenia (899.83 ± 661.71 vs 673.13 ± 465.88 ng/ml, p = 0.038); we also found a positive correlation with erythrocyte sedimentation rate values (Pearson coefficient 0.163, p = 0.076) and with serum gammaglobulin levels (Pearson coefficient 0.26, p = 0.005). We retrospectively analyzed in a subset of patients the correlation between SP-D levels and serum immunological markers, including serum immunoglobulins IgG, IgM, and IgA, C-reactive protein, β_2-microglobulin, soluble selectin, serum CD5 and CD6, and cytokines [interleukin (IL)-2, IL-6, IL-10, and tumor necrosis factor (TNF)-α]. A

significant correlation was only found for IgG levels (p = 0.012) and statistically significant trends with serum CD5 (p = 0.113), TNF-α (p = 0.12), and soluble selectin (p = 0.112) levels. With respect to autoantibodies, higher SP-D levels were found in patients with positive rheumatoid factor (RF; 851.23 ± 677.74 vs 663.94 ± 411.43 ng/ml, p = 0.064), anti-Ro/SS-A antibodies (927.26 ± 731.29 vs 642.75 ± 377.23 ng/ml, p = 0.006), and anti-La/SS-B antibodies (933.28 ± 689.63 vs 650.41 ± 428.14 ng/ml, p = 0.007). After correction for multiple comparisons, SP-D levels correlated with renal involvement and anti-Ro/La antibodies.

Table 4 summarizes the mean values of serum SP-D according to the development of the main adverse outcomes during the followup after blood extraction. Patients who developed hyperuricemia (1133.35 ± 928.96 vs 707.44 ± 481.66 ng/ml, p = 0.047) and infections (896.31 ± 736.24 vs 688.93 ± 453.25 ng/ml, p = 0.076) had higher serum SP-D levels.

DISCUSSION

To our knowledge, ours is the first study to evaluate the relationship between genetic polymorphisms and serum levels of SP-D in patients with pSS, one of the most prevalent systemic autoimmune diseases. SP-D is involved in innate immune responses and has been investigated in patients with other systemic autoimmune diseases, including systemic sclerosis (SSc), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA)[19,20,21,22,23]. Our

Table 4. Cardiovascular risk factors and outcomes of patients with pSS according to the SP-D serum levels.

Characteristics	SP-D levels, ng/ml, mean ± SD	Bilateral p
Cardiovascular risk factors		0.729
Yes	744.92 ± 552.41	
No	702.92 ± 468.69	
Hyperuricemia		0.047
Yes	1133.35 ± 928.96	
No	707.44 ± 481.66	
Metabolic syndrome		0.896
Yes	747.62 ± 466.72	
No	732.40 ± 555.77	
Cardiovascular disease		0.169
Yes	581.21 ± 286.81	
No	765.39 ± 565.54	
Infections		0.076
Yes	896.31 ± 736.24	
No	688.93 ± 453.25	
Neoplasia		0.214
Yes	860.82 ± 691.87	
No	706.08 ± 488.93	
Death		0.588
Yes	803.64 ± 513.97	
No	725.48 ± 539.05	

pSS: primary Sjögren syndrome; SP-D: surfactant protein-D.

results showed that SP-D serum levels had a stronger association with disease expression than Met11Thr polymorphisms, suggesting a poor correlation between SP-D polymorphisms and serum levels in pSS.

We found no significant differences in the prevalence of the Met11Thr polymorphisms of the SP-D gene between patients with pSS and controls, whose percentages were also similar to those found in healthy controls included in previous studies in European populations (Met11/Met11 35%, Thr11/Thr11 18%, Met11/Thr11 46%)[23]. With respect to the clinical significance of the Met11Thr polymorphisms in patients with pSS, we found no correlation with most epidemiological, clinical, and immunological SS features, except for a higher frequency of renal involvement in patients carrying the Thr11/Thr11 genotype. Other studies in autoimmune diseases have also found a poor correlation between SP-D genotypes and clinical disease expression in patients with RA[22,23] and SLE[21]. In contrast, some studies have correlated genetic variants with poor outcomes in patients with pulmonary diseases[24,25].

The mechanisms involved in the synthesis of SP-D are unknown, but seem to be genetically influenced. In healthy subjects, some studies have shown that the Thr11/Thr11 genotype is associated with SP-D deficiency[11,12]. In our patients with pSS, we found no significant correlation between genetic polymorphisms and serum SP-D levels. Some authors have suggested that the association with serum levels from a single polymorphism may underestimate the genetic association, which could be represented by haplotype blocks[26]. In addition, Sorensen, *et al*[11] found that serum SP-D is influenced not only by the effect of Met11Thr variations, but also by additive genetic effects and environmental factors while other studies have found that serum SP-D levels increased with male sex, age, smoking status, and physical activity[27]. In addition, oligomerization of SP-D may be influenced by the Met11Thr polymorphism, and studies have suggested a differentiated role in the immune response of SP-D multimers (antiinflammatory) with respect to SP-D trimers (proinflammatory)[28,29,30]. It could be hypothesized that, in pSS, genetic polymorphisms may have an influence on the etiopathogenic local epithelial process while serum SP-D levels may reflect the enhanced inflammatory systemic response seen in patients with greater clinical and immunological activity.

The clinical utility of measuring serum SP-D levels is centered on the correlation between high levels and chronic pulmonary diseases or cancer[31,32,33]. Some studies have also evaluated SP-D levels in patients with autoimmune and rheumatic systemic diseases and have found lower levels of SP-D in patients with SLE (mean of 800 ng/ml)[21] and RA (mean 693–878 ng/ml)[22,23] in comparison with healthy subjects, who have a mean of > 900 ng/ml[21,23]. Our study found lower levels of SP-D in patients with pSS (mean of 734 ng/ml) as reported in patients with SLE and RA. In

contrast, higher SP-D levels have been reported in patients with SSc[19,20,34,35], especially those with pulmonary involvement. Therefore, it could be postulated that SP-D levels are higher in autoimmune diseases with a higher frequency of interstitial pulmonary involvement, and lower in diseases in which the majority of patients have no pulmonary inflammatory involvement.

Thus, the rationale for correlating SP-D levels with chronic pulmonary diseases (even those with a different etiology) seems clear because, as mentioned, SP-D is synthesized primarily in the lungs. SP-D has been linked to the maintenance of the equilibrium of the pulmonary immune system, and plays a double role as both an antiinflammatory agent in basal conditions and as a promoter of inflammation in clearance of pathogens[6,36,37,38]. It has also been associated with a potential antitumoral effect[39] and with other spliced proteins involved in mucosal innate immunity, such as DMBT1[40]. Other immune-related functions of SP-D include cytokine secretion, clearance of apoptotic cells, or reduction of autoantibody generation in mice models[41,42,43]. An association has been observed between pulmonary diseases and increased serum levels of SP-D, which correlate with disease activity[44,45] in patients with idiopathic ILD[46], hepatitis C virus infection treated with interferon[47], and in patients with SSc, especially those with pulmonary involvement[19,20,21,36,37,48]. Our results show that, in spite of the low mean levels found in our patients with pSS, those with SS-related ILD had elevated serum levels of SP-D (> 1000 ng/ml) as reported in patients with SSc-related ILD, suggesting that measurement of serum SP-D could be a potentially useful soluble biomarker in clinical practice. Interestingly, we found an inverse correlation between SP-D levels and bronchiectasis, another frequent pulmonary disease in patients with pSS[49]. A possible explanation could be the frequent asymptomatic presentation of bronchiectasis in pSS in contrast to ILD, and also the different etiopathogenic role of SP-D, which has been clearly shown to be involved in the remodeling of an animal model of lung fibrosis[50].

The finding that SP-D is expressed in other extrapulmonary tissues suggests that it might play a role in the development of autoimmune damage in other organs. Our results show that patients with SS-related renal involvement had higher serum SP-D levels. The presence of SP-D in glomeruli and renal tubules[7] has been described. A study by Hu, *et al*[51] found that SP-D functions as an antiinflammatory factor in renal tubular epithelial cells and may modulate tubulointerstitial fibrosis in the kidney. Xie, *et al*[52] correlated serum SP-D levels with the prognosis in patients with chronic renal disease. These studies, together with our findings in pSS, suggest that SP-D could play a role in renal involvement because of its potential proinflammatory role in the tubular epithelial cells.

Gardai, *et al*[36] found that SP-D may induce either pro-

inflammatory or antiinflammatory responses, depending on the orientation of the molecule. This dual role of SP-D is important in maintaining homeostasis in the lungs through suppression of inflammation in naive healthy lungs and through induction of inflammation when active clearance of damaging pathogens, allergens, and apoptotic and necrotic cells is required[37]. In systemic inflammation, it seems that serum SP-D may act as an acute-phase reactant. In systemic autoimmune diseases, it could be hypothesized that high serum SP-D levels may be associated to a nonspecific systemic inflammation (consequence of the immune system activation) or may be closely related to the pathogenesis of the disease. In our study, we found that high serum SP-D levels were more closely associated with B cell hyperactivity (hypergammaglobulinemia, raised IgG levels, and positive RF, anti-Ro, and anti-La antibodies) and less with acute reactant proteins.

The etiopathogenic role of SP-D in pSS seems to be centered on peripheral serum levels, with genetic polymorphisms having little influence. Patients with greater glandular and extraglandular involvement had higher SP-D serum levels, as did patients carrying anti-Ro/La autoantibodies. Serum SP-D levels may have a potential role as a soluble marker of systemic disease activity in patients with pSS, especially those with interstitial pulmonary and renal involvement.

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Gene Variation at Immunomodulatory and Cell Adhesion Molecules Loci Impacts Primary Sjögren's Syndrome

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Primary Sjögren's syndrome (pSS) is an autoimmune disease triggered by a combination of environmental and host genetic factors, which results in the focal lymphocytic infiltration of exocrine glands causing eye and mouth dryness. Glandular infiltrates include T and B cell subsets positive for CD5 and/or CD6, two surface scavenger receptors involved in the fine-tuning of intracellular signals mediated by the antigen-specific receptor complex of T (TCR) and B (BCR) cells. Moreover, the epithelial cells of inflamed glands overexpress CD166/ALCAM, a CD6 ligand involved in homo and heterotypic cell adhesion interactions. All this, together with the reported association of functionally relevant single nucleotide polymorphisms (SNPs) of *CD5*, *CD6*, and *CD166/ALCAM* with the risk or prognosis of some immune-mediated inflammatory disorders, led us to investigate similar associations in a local cohort of patients with pSS. The logistic regression analyses of individual SNPs showed the association of *CD5* rs2241002[T] with anti-Ro/La positivity, *CD6* rs17824933[C] with neutropenia, and *CD6* rs11230563[T] with increased leukopenia and neutropenia but decreased peripheral nervous system EULAR Sjögren's syndrome disease activity index (ESSDAI). Further analyses showed the association of haplotypes from *CD5* (rs2241002[T]-rs2229177[C]) with anemia and thrombocytopenia, *CD6* (rs17824933[G]-rs11230563[C]-rs12360861[G]) with cutaneous ESSDAI, and *CD166/ALCAM* (rs6437585[C]-rs579565[A]-rs1044243[C] and rs6437585[C]-rs579565[G]-rs1044243[T]) with disease susceptibility and several analytical parameters (anti-nuclear antibodies, neurological ESSDAI, and hematologic cytopenias). These results support the relevance of gene variation at loci coding for cell surface receptors involved in the modulation of T and B lymphocyte activation (*CD5*, *CD6*) and epithelial-immune cell adhesion (*CD166/ALCAM*) in modulating the clinical and analytical outcomes in patients with pSS.

Keywords: Sjögren's syndrome, *CD5*, *CD6*, CD166/ALCAM, polymorphism, SNP

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INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic, systemic rheumatic disease characterized by the lymphoplasmacytic infiltration of exocrine glands—mainly salivary and lacrimal glands—resulting in sicca syndrome and systemic manifestations (1). It is a common disorder (prevalence of 0.5–1% in the general population) with a female/male ratio of approximately 9:1 (2, 3). pSS is considered as a complex and multifactorial process whose pathogenesis involves environmental factors, such as viral infections, combined with sex hormonal, genetic and epigenetic factors, causing epithelial cell barrier disruption followed by an abnormal immune cell-mediated inflammatory response (4, 5).

Periductal immune cell infiltrates in the affected glands of patients with pSS include CD5- and/or CD6-positive T and B cells (6–9). CD5 and CD6 are two highly homologous lymphocyte surface receptors of the scavenger receptor cysteine-rich superfamily (SRCR-SF) (10). They are expressed on all T cells and a subset of B cells (B1a) involved in the production of polyreactive natural antibodies, and they are abnormally expanded in the peripheral blood of patients undergoing autoimmune disorders, such as pSS and systemic lupus erythematosus (SLE) (6, 10, 11). Both receptors are signal-transducing molecules that modulate intracellular activation and differentiation signals from the antigen-specific receptor complex of T (TCR) and B (BCR) cells to which CD5 and CD6 physically associate (12–14). In addition, CD5 and CD6 act as pattern recognition receptors (PRRs) by recognizing microbial-associated molecular patterns (MAMPs) from the bacterial, fungal, viral, and parasitic origin (15–17). Particularly, CD5 has been shown to interact with fungal β-glucans (18), hepatitis C virus (19), and tegumental structures of *Echinococcus granulosus* (20), while CD6 interacts with lipopolysaccharide, lipoteichoic acid, and peptidoglycan from Gram-negative and -positive bacteria (21), gp120 from human immunodeficiency virus 1 (22), and the tegumental components of *E. granulosus* (20).

A central phenomenon in the immunopathogenesis of pSS is the aberrant epithelial cell activation status (pSS has been described as an autoimmune epithelitis) (23, 24). This results in the increased expression of human leukocyte antigen (HLA)-DR, costimulatory, and adhesion molecules. Among the latter, overexpression of the well-known CD6 ligand CD166/ALCAM has been reported in pSS epithelial lesions (8, 9, 25). CD166/ALCAM (for activated leukocyte cell adhesion molecule) is an adhesion molecule of the immunoglobulin superfamily with a broad tissue distribution, such as epithelia, endothelia, neurons, myeloid progenitors, hematopoietic stem cells, mesenchymal stem cells, bone marrow stromal cells, and cancer cells (26). Interestingly, CD166/ALCAM establishes not only homophilic (ALCAM-ALCAM) but also higher affinity heterophilic (ALCAM-CD6) interactions with the CD6 lymphocyte receptor, which facilitate cell interactions of T or B1a lymphocytes with epithelial and endothelial cells (26–28).

Studies aimed at the genetic basis of pSS show the associations of both human leukocyte antigen (HLA) and non-HLA genes with pSS susceptibility. The HLA-DR and HLA-DQ alleles have shown the strongest associations across different ethnicities (29, 30). The long, though still incomplete, list of non-HLA genetic polymorphisms contributed by genome-wide (GWAS) and gene-driven association studies includes interferon regulatory factor 5 (IRF5), signal transducer and activator of transcription 4 (STAT4), B lymphocyte kinase (BLK), tumor necrosis factor-α (TNF-α), interleukin (IL)-4, IL-10, IL-12A, C-X-C chemokine receptor type 5 (CXCR5), surfactant protein-D (SP-D), and Mannan-binding lectin (MBL) (30–36).

Single nucleotide polymorphisms (SNPs) at the *CD5*, *CD6*, and *CD166/ALCAM* gene loci have been associated with different immune-mediated inflammatory diseases (IMID) (37). Specifically, *CD5* variation has been associated with rheumatoid arthritis (RA) susceptibility (38) and the development of lupus nephritis (39). *CD6* and *CD166/ALCAM* SNPs have been identified and validated as risk factors for the development and progression of multiple sclerosis (MS) (40–42), psoriasis severity (43), Behçet's disease risk (44), and inflammatory bowel disease (IBD) risk (45, 46).

Given the expression of CD5, CD6, and CD166/ALCAM in pSS inflamed tissue and the association of their SNPs with other IMIDs, we hypothesize that variation at *CD5*, *CD6*, and *CD166/ALCAM* loci may impact the pathology of pSS. The results of the present candidate gene-driven association analysis show that *CD5*, *CD6*, and *CD166/ALCAM* genetic polymorphisms are associated with the clinical and analytical parameters of the disease in a local cohort of pSS patients.

MATERIALS AND METHODS

Subjects

Consecutive patients with pSS ($n = 212$) attending to the Hospital Clínic de Barcelona, Barcelona, Spain were included in the study (**Table 1**). Patients fulfilled the 2002/2016 criteria approved by the American-European Consensus Group (47). Exclusion criteria for considering SS as a primary disease were chronic HCV/HIV infection, previous lymphoproliferative processes, and associated systemic autoimmune diseases. Diagnostic tests for SS (ocular tests, parotid scintigraphy, and salivary gland biopsy) were performed according to the European Community Study Group recommendations (48).

Unrelated volunteers ($n = 305$) from the Banc de Sang i Teixits (BST) from Generalitat de Catalunya were included as controls (143 women and 162 men).

The study was approved by the local Hospital Ethics Committee, and written informed consent was obtained from all participants before inclusion and blood extraction.

Definition of Variables

Disease diagnosis was defined as the time when the attending physician confirmed the fulfillment of the 2002/2016 criteria (47). The main disease features were retrospectively collected and analyzed. The following clinical variables were selected for harmonization and further refinement: age, gender, ethnicity, country of residence, fulfillment of the 2002/2016 criteria items, antinuclear antibodies (ANA), rheumatoid factor (RF), C3 and C4 levels, and cryoglobulins. The epidemiological variables included in this study were age at diagnosis, gender, and

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TABLE 1 | General characteristics of the primary Sjögren's syndrome (pSS) cohort.

Variables	n (%)
Gender (Female)	202 (95.3)
Ethnicity (Caucasian)	201 (94.8)
Age at diagnosis	54 (14.4)
Dry mouth	212 (100)
Dry eyes	205 (96.7)
Schirmer test (abnormal)	185/194 (95.4)
Salivary scintigrahy (abnormal)	163/180 (90.6)
Minor salivary gland biopsy (positive)	103/113 (91.2)
Antinuclear antibodies (positive)	181/211 (85.8)
Rheumatoid factor (positive)	98/208 (47.1)
Anti-Ro/La antibodies (positive)	151 (71.2)
Anti-Ro	143 (67.5)
Anti-La	103/211 (48.8)
Monoclonal gammopathy	25/142 (17.6)
Low C3 levels (<0.82 g/L)	19/210 (9)
Low C4 levels (<0.11 g/L)	13/207 (6.3)
Cryoglobulins	17/201 (8.5)
Cytopenias	109/211 (51.7)
Anemia (Hb < 110 g/L)	43/211 (20.4)
Leukopenia (<4,000/mm^3)	57/211 (27)
Thrombocytopenia (<150,000/mm^3)	23/211 (10.9)
Neutropenia (<1,500/mm^3)	53/211 (25.1)
Lymphopenia (<1,000/mm^3)	21/211 (10)
ESSDAI domains (activity)	
Constitutional	28 (13.2)
Lymphadenopathy	27 (12.7)
Glandular	60 (28.3)
Articular	93 (43.9)
Cutaneous	37 (17.5)
Pulmonary	41 (19.3)
Renal	5 (2.4)
Muscular	1 (0.5)
Peripheral nervous system	23 (10.8)
Central nervous system	8 (3.8)
Hematological	159 (75)
Biological	141 (66.5)
Total ESSDAI (baseline)	7.4 (6.8)
Total ESSDAI (cumulative)	10.2 (8.5)

ethnicity according to the Food and Drug Administration (FDA) definitions (49). Systemic involvement at diagnosis was retrospectively classified and scored according to the EULAR Sjögren's syndrome disease activity index (ESSDAI) (50), which evaluates 12 domains or organ systems, and the ClinESSDAI (51), which evaluates the same domains but excluding the last (biological) domain. Each domain is divided into 3–4 levels according to the degree of activity and scored as 0 (no activity), 1 (low activity), 2 (moderate activity), or 3 (high activity) (52). Disease activity states (DAS) were calculated as: no activity (global score = 0), low activity (global score 1–4), moderate

activity (global score 5–13), and high activity (global score ≥14) (53).

Additionally, cumulative systemic involvement was classified and scored according to the ESSDAI. Cumulative systemic involvement was defined as the systemic activity present since the diagnosis of pSS to the last medical visit.

Genotyping

DNA was purified from ethylenediaminetetraacetic acid (EDTA)-treated peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Venio, The Netherlands) and subjected to real-time (RT)-PCR with the following TaqMan probes: CD5 rs2241002 (assay number: C__25472293_20), CD5 rs2229177 (assay number: C___3237272_10), CD6 rs17824933 (assay number: C__33967506_10), CD6 rs11230563 (assay number: C__31727142_10), CD6 rs12360861 (assay number: C__25922320_10), and CD166/ALCAM rs6437585 (assay number: C__29281365_20), all from ThermoFisher Scientific (Barcelona, Spain). Primers for PCR amplification and further sequence-base typing (PCR-SBT) of CD166/ALCAM rs579565 and rs1044243, which lie 2 bp apart from each other, were also from ThermoFisher Scientific (Hs00666884_CE assay). SNP genotyping and clinical data are available at a public repository (54).

Statistical Analyses

Statistical analyses were performed with R 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria). Genotypic statistical associations among the SNPs and susceptibility or disease outcomes were tested by generalized linear models using the R package "SNPstats." For each analysis, 4 models were generated (codominant, dominant, recessive, and log-additive), and the model with the lowest Akaike information criterion (AIC) was chosen. The p values were corrected for false discovery rate (FDR, q values). Haplotypic analyses were performed with generalized linear models by means of the R package "haplo.stats."

RESULTS

A total of 212 patients with pSS with a mean age of 54 years at diagnosis were included in the study, most of them were women (95.3%) and presented dry mouth (100%) and dry eyes (96.7%). The association of individual SNPs with susceptibility and the clinical parameters of pSS was first investigated (**Supplementary Table 1**). Sex is a major risk factor in pSS, so statistical models for subphenotypical analyses were generated with or without including sex as a covariant, and their goodness of fit compared with the AIC. The results presented here do not include sex as a covariant, as these models had lower AIC. Susceptibility analyses were performed only with female patient cases and controls. No significant association was found between any individual CD5, CD6, and CD166/ALCAM SNPs and pSS susceptibility, although the CD166/ALCAM rs579565[A] allele showed a trend for statistical association in women ($q =$ 0.064) (**Table 2**).

Regarding association with pSS clinical parameters, the CD5 rs2241002[C] allele was found associated with a higher frequency of

TABLE 2 | Logistic regression analyses of *CD166/ALCAM* SNP association with pSS susceptibility.

Gene	SNP	Model	Genotype	Controls (%)	pSS cases (%)	OR (95% CI)	q value
CD166/ ALCAM	rs579565	Recessive	G/G-G/A	139 (97.9)	169 (91.4)		0.064
			A/A	3 (2.1)	16 (8.6)	4.39 (1.25, 15.36)	

TABLE 3 | Logistic regression analyses of *CD5* and *CD6* SNPs association with anti-Ro/La antibodies, neutropenia, leukopenia, and peripheral nervous system (PNS) EULAR Sjögren's syndrome disease activity index (ESSDAI) activity.

Gene	SNP	Model	Genotype	No anti-Ro/La (%)	Anti-Ro/La (%)	OR (95% CI)	q value
CD5	rs2241002	Recessive	C/C-C/T	55 (90.2)	149 (98.7)		0.046
			T/T	6 (9.8)	2 (1.3)	0.12 (0.02, 0.63)	
				No neutropenia (%)	**Neutropenia (%)**		
CD6	rs17824933	Dominant	C/C	75 (50.3)	33 (73.3)		0.022
			C/G-G/G	74 (49.7)	12 (26.7)	0.37 (0.18, 0.77)	
				No leukopenia (%)	**Leukopenia (%)**		
	rs11230563	Recessive	C/C-C/T	121 (85.8)	34 (65.4)		0.019
			T/T	20 (14.2)	18 (34.6)	3.20 (1.53, 6.73)	
				No neutropenia (%)	**Neutropenia (%)**		
		Recessive	C/C-C/T	127 (85.8)	28 (62.2)		0.008
			T/T	21 (14.2)	17 (37.8)	3.67 (1.72, 7.84)	
				No PNS ESSDAI activity (%)	**PNS ESSDAI activity (%)**		
		Dominant	C/C	50 (28.7)	12 (60.0)		0.041
			C/T-T/T	124 (71.3)	8 (40.0)	0.27 (0.10, 0.70)	

anti-Ro/La antibody positivity (**Table 3**). The *CD6* rs17824933[G] allele was associated with decreased risk of neutropenia (**Table 3**), and the *CD6* rs11230563[T] allele with increased leukopenia and neutropenia, but decreased ESSDAI peripheral nervous system (PNS) activity (**Table 3**).

Haplotypic analyses showed the association of *CD5* rs2241002[T]-rs2229177[C] haplotype with an increased risk of anemia and thrombocytopenia (**Table 4**). The *CD6* rs17824933[G]-rs11230563[C]-rs12360861[G] haplotype was associated with an increased risk of ESSDAI cutaneous activity (**Table 5**). The *CD166/ALCAM* rs6437585[C]-rs579565[G]-rs1044243[T] haplotype was associated with increased ANA positivity, ESSDAI PNS activity, and hematologic cytopenias, such as anemia and lymphopenia (**Table 6**).

Case-control analyses to assess the influence of *CD5*, *CD6*, and *CD166/ALCAM* haplotypes on pSS risk were also performed. To account for the gender skew in pSS, only female cases and controls were included in this haplotypic analysis. The results showed that the only associations with pSS susceptibility were with the *CD166/ALCAM* rs643785[C]-rs579565[A]-rs1044243[C] (CAC) and rs643785[C]-rs579565[G]-rs1044243[T] (CGT) haplotypes (**Table 6**), which were over-represented in the case cohort,

indicating the association of rs579565[A] and rs1044243[T] alleles with pSS susceptibility.

DISCUSSION

The pathophysiology of pSS is complex and multifactorial. How the innate and adaptive immune responses are dysregulated through both cellular- and humoral-mediated processes (30) is still poorly understood. Identifying genetic factors associated with pSS may help in the better comprehension of pathogenic mechanisms leading to the overall pSS phenotype and clinically heterogeneous subsets of patients (55). By using a candidate gene-driven strategy, the present work shows evidence on the impact of *CD5*, *CD6*, and *CD166/ALCAM* gene variants in the susceptibility and clinical expression of pSS, thus supporting their involvement in pSS pathophysiology.

CD5, *CD6*, and *CD166/ALCAM* variation study in pSS responds to: first, the three genes encode functionally relevant and related cell surface receptors. CD5 and CD6 are highly homologous lymphocyte receptors of the ancient and highly conserved SRCR-SF and are encoded by contiguous genes likely resulting from a duplication event (56, 57). Both CD5 and

TABLE 4 | Logistic regression analysis of CD5 haplotype association to anemia and thrombocytopenia.

Haplotype			Anemia			
rs2241002	rs2229177	% in cohort	% No	% Yes	p value	OR (95% CI)
C	C	41.0	41.8	39.9		
C	T	39.1	38.9	38.0	0.941	1.02 (0.58, 1.78)
T	T	15.2	16.5	12.0	0.399	0.69 (0.29, 1.62)
T	**C**	**4.7**	**2.8**	**10.0**	**0.032**	**4.48 (1.14, 17.60)**
		% in cohort	Thrombocytopenia			
			% No	% Yes		
C	C	41.0	39.7	35.9		
C	T	39.1	41.4	35.9	0.905	1.07 (0.46, 2.50)
T	T	15.2	15.1	14.1	0.872	0.93 (0.27, 3.19)
T	**C**	**4.7**	**3.8**	**14.1**	**0.036**	**5.83 (1.12, 30.29)**

Significant haplotypes are shown in bold.

TABLE 5 | Logistic regression analysis of CD6 haplotype association to cutaneous affectation. Only the 4 most common haplotypes are shown.

Haplotype				Cutaneous ESSDAI activity			
rs17824933	rs11230563	rs12360861	% in cohort	% No	% Yes	p value	OR (95% CI)
C	C	G	32.6	35.2	20.1		
G	**C**	**G**	**23.6**	**22.1**	**31.4**	**0.012**	**2.85 (1.26, 6.43)**
C	T	A	22.4	22.3	20.8	0.141	1.81 (0.82, 4.00)
C	T	G	20.6	20.0	25.8	0.055	2.26 (0.98, 5.12)

Significant haplotypes are shown in bold.

CD6 are expressed by all T cell types and the B1a cell subset, with the lower levels of expression in other cell types (e.g., macrophages, dendritic cells, or natural killer cells) (10, 13), all found in pSS periductal immune cell infiltrates (6–9). From the functional point of view, CD5 and CD6 are considered relevant signaling immune receptors at the interphase of the innate and adaptive immune responses as a result from their involvement in (i) the recognition and sensing of bacterial, viral, and/or parasitic MAMPs (17) and (ii) the fine-tuning of lymphocyte activation signals delivered by clonotypic T and B antigen-specific receptors, which they are physically associated to (58–60). While the nature of the endogenous CD5 ligand is yet uncertain, one of the most-well studied CD6 ligands is CD166/ALCAM, a cell adhesion molecule overexpressed in pSS salivary gland epithelial cells (8, 9, 25), but also RA synovium (61), MS blood–brain barrier endothelium (62), and lupus nephritis kidneys (63), thus contributing to T and B cell migration and infiltration at inflamed tissues in autoimmune processes.

Second, several *CD5*, *CD6*, and/or *CD166/ALCAM* gene variants have been associated with different IMIDs, such as RA (38), lupus nephritis (39), MS (40–42), psoriasis (43), Behçet's disease (44), and IBD (45, 46) (**Supplementary Table 2**). The *CD5*, *CD6*, and *CD166/ALCAM* SNPs included in the present study were selected not only for being informative in the above-mentioned IMIDs but also for their putative

functional relevance. Regarding CD5, the rs2241002 (C > T) and rs2229177 (C > T) SNPs result in amino acid substitutions at the extracellular SRCR2 domain (Pro224>Leu) and just next to a cytoplasmic ITAM-like motif (Ala471>Val), respectively (39, 64). Functional studies show that homozygous carriers for the ancestral rs2241002^C-rs2229177^C haplotype (Pro224-Ala471) present increased T-cell proliferation and cytokine release and a bias toward a Th2 profile, compared with the homozygous carriers of more recently derived rs2241002^C-rs2229177^T haplotype (Pro224-Val471) (39). Regarding CD6, the rs11230563 (C>T) and rs12360861 (G>A) SNPs result in amino acid substitutions at the extracellular SRCR2 (Arg225>Trp) and SRCR3 (Ala271>Thr) domains, respectively, and the intron 1 rs17824933 (C>G) SNP results in the skipping of exon 5 and expression of a CD6 isoform lacking the SRCR3 domain (CD6Δd3), in which the CD166/ALCAM-binding site locates (65). Functional studies show that the *CD6* rs11230563^C-rs2074225^C haplotype (Arg225-Ala257) results in higher CD6 surface expression on CD4$^+$ and CD8$^+$ naïve T cells and NKT cells (41). The carriage of *CD6* rs17824933^G allele results in an increased CD6Δd3/full-length CD6 ratio driving to lower CD4$^+$ T cell activation responses (66). Regarding *CD166/ALCAM*, the rs6437585 (C > T) SNP maps at the 5'-untranslated region (UTR) and is known to influence the transcriptional activity of *CD166/ALCAM* (42, 67), while the rs579565 (G > A) and rs1044243 (C > T) SNPs result in synonymous

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TABLE 6 | Logistic regression analysis of *CD166/ALCAM* haplotype association with anti-nuclear antibodies (ANA), cytopenia, anemia, lymphopenia, peripheral nervous system (PNS) ESSDAI activity, and pSS susceptibility.

Haplotype				ANA positivity		p value	OR (95% CI)
rs6437585	rs579565	rs1044243	% in cohort	% Negative	% Positive		
C	G	C	55.1	58.4	54.3		
C	A	C	27.3	30.5	27.2	0.685	0.87 (0.46, 1.66)
C	**G**	**T**	**13.0**	**3.7**	**14.3**	**0.045**	**4.64 (1.04, 20.81)**
T	G	C	3.0	4.5	3.0	0.494	0.57 (0.11, 2.90)

				Cytopenia			
			% in cohort	% No	% Yes		
C	G	C	55.4	58.0	53.1		
C	A	C	27.1	27.8	26.3	0.975	0.97 (0.61, 1.54)
C	**G**	**T**	**12.8**	**9.0**	**16.5**	**0.027**	**2.14 (1.08, 4.21)**
T	G	C	3.0	3.5	3.0	0.657	0.71 (0.16, 3.15)

				Anemia			
			% in cohort	% No	% Yes		
C	G	C	55.4	57.1	49.0		
C	A	C	27.1	26.7	27.6	0.632	1.15 (0.65, 2.06)
C	**G**	**T**	**12.8**	**11.0**	**20.7**	**0.030**	**2.25 (1.08, 4.66)**
T	G	C	3.0	3.6	0.0	–	–

				Lymphopenia			
			% in cohort	% No	% Yes		
C	G	C	55.4	56.1	56.3		
C	A	C	27.1	27.4	18.8	0.907	0.95 (0.44, 2.08)
C	**G**	**T**	**12.8**	**11.3**	**18.8**	**0.030**	**2.64 (1.10, 6.35)**
T	G	C	3.0	3.2	0.0	–	–

				PNS ESSDAI activity			
			% in cohort	% No	% Yes		
C	G	C	55.1	55.6	52.5		
C	A	C	27.3	28.1	20.0	0.404	0.70 (0.30, 1.62)
C	**G**	**T**	**13.0**	**11.5**	**25.0**	**0.036**	**2.56 (1.06, 6.15)**
T	G	C	3.0	3.1	0.0	–	–

				pSS susceptibility			
			% in pool	% controls	% cases		
C	G	C	58.5	63.3	54.8		
C	**A**	**C**	**24.4**	**20.6**	**27.3**	**0.044**	**1.51 (1.01, 2.24)**
C	**G**	**T**	**11.4**	**8.8**	**13.3**	**0.046**	**1.72 (1.01, 2.95)**
T	G	C	4.2	5.8	2.7	0.274	0.62 (0.26, 1.47)

Only the 4 most common haplotypes are shown. Significant haplotypes are shown in bold.

(Leu300>Leu) and non-synonymous (Thr301>Met) changes at the extracellular C1-like domain (42) with still unknown functional consequences.

Individual SNP and haplotypic analyses showed the association of *CD5*, *CD6*, and *CD166/ALCAM* SNPs with different pSS clinical parameters. Thus, the *CD5* rs2241002^C allele and the minor *CD5* rs2241002^T-rs2229177^C haplotype, previously associated with a more aggressive form of SLE (lupus nephritis) (39), showed association with anti-Ro/anti-La antibody positivity, and with anemia and thrombocytopenia, respectively. This could be interpreted as a result of hyperactive autoantibody-producing B cells (most likely CD5$^+$ B1a cells) in pSS carriers of such *CD5* variants.

The individual *CD6* rs11230563^C allele was associated with the higher risk of PNS ESSDAI activity, and the *CD6* rs17824933^G-rs11230563^C-rs12360861^G haplotype with cutaneous ESSDAI activity. This is reminiscent of the increased MS risk and psoriasis severity previously reported for rs11230563^C allele (40, 43, 68–70). It is noteworthy that both rs17824933^G and rs11230563^C alleles were associated with the reduced risk of neutropenia. Since both alleles impact the extracellular region of CD6 (an increased expression of CD6Δd3 isoform and Arg225 to Trp substitution at SRCR2, respectively), it remains to be analyzed whether this relates to the reported surface CD6 (and CD166/ALCAM) expression by hematopoietic cell progenitors present in the bone marrow and in mobilized blood (71, 72).

The *CD166/ALCAM* (rs6437585^C-rs579565^G-rs1044243^T) haplotype was found associated with the increased incidences of ANA positivity, neurological affectation, and hematologic cytopenias. These results further support the damaging role of *CD6* rs17824933^G and rs11230563^C alleles and of *CD166/ALCAM* rs1044243^T allele by worsening some analytical and clinical parameters of pSS. Interestingly, haplotypic analyses showed the association of *CD166/ALCAM* rs6437585^C-rs579565^A-rs1044243^C and rs6437585^C-rs579565^G-rs1044243^T haplotypes with increased pSS susceptibility. This supports a role for minor rs579565^A and rs1044243^T alleles in pSS susceptibility, which is reminiscent of the earlier age of MS diagnosis reported for the rs579565^A allele (42).

The association of *CD5*, *CD6*, and *CD166/ALCAM* SNPs with pSS phenotype highlights the relevance of genetic variation at loci related with immune activation in pSS pathophysiology. In addition, this is illustrated by the previously reported association of HLA-DR and HLA-DQ, *IRF5*, *STAT4*, *BLK*, *TNF*, *IL4RA*, *IL10*, *IL12A*, *CXCR5*, *TNFAIP3*, *MTHFR*, *CD28*, *CTLA4*, *IKZF1*, *HIF1A*, *AKNA*, *SFTPD*, and *MBL2* loci with pSS (30–36, 73, 74) (**Supplementary Table 3**). Interestingly, CD5 and CD6 interact with microorganisms, such as SP-D and mannose-binding lectin (encoded by *SFTPD* and *MBL*, respectively). This brings out the relevance of microbial/pathogen recognition in pSS.

We are aware of some limitations in the present study regarding: first, the limited number of pSS cases and controls in this single-center study. Second, only a single patient cohort was available for the analysis in spite of our efforts to access validation cohorts with the necessary subphenotypical data for replicates. Therefore, validation in an independent cohort is pending for significant confirmation of the role of *CD5*, *CD6*, and *CD166/ALCAM* gene variants in pSS.

In summary, we identified the *CD166/ALCAM* rs579565 and rs1044243 SNPs as pSS risk markers, and the *CD5* rs2241002, *CD6* rs17824933 and rs11230563 and *CD166/ALCAM* rs1044243 SNPs as disease modifiers markers. Further studies in independent cohorts will be required to validate these results. Nevertheless, our observations are the first to support a role for *CD5*, *CD6*, and *CD166/ALCAM* variation in pSS, and they highlight the shared immunogenetic basis of different IMIDs (75). These results, along with the identification of other genetic factors involved in pSS etiopathogenesis, may also help to classify patients and allow better identification, management, and treatment of the disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://github.com/SergiCLl/CD5-CD6-ALCAM-pSS, CD5-CD6-ALCAM-pSS.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitè d'Ètica de la Investigació amb medicaments (CEIm) Hospital Clínic de Barcelona. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FL, MR-C, and PB-Z conceptualized the study. SC-L, HG, SA, MC-F, and NA-B contributed to genetic studies. HG, MR-C, and PB-Z contributed to sample and clinical information collection. SC-L and BK contributed to statistical analyses. SC-L, PB-Z, and FL wrote the original draft. All authors read, critically revised, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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DISCUSSION

FIGURE 6. Summary of the main results of the thesis.

These results will be discussed in the next pages.

Study of posible factors related to the etiopathogenesis of Sjögren syndrome

	HCV	SP-D Serum levels	SP-D SNPs	CD5 SNPs	CD6 SNPs	ALCAM-CD166 SNPs
Epidemiological	Men	NC	NC	NC	NC	Women^^
Clinical	Cryo-related	↑Parotid involv. ↑ ILD ↑Renal ↓ bronchiectasias	↑Renal		Skin＋ PNSλ	PNS^
Analytical		Leucopenia	NC	Anemia* Low platelets*	NeutropeniaФ	Anemia^ Lymphopenia^
ANA	↓	NC	NC	NC	NC	↑^
Anti-Ro	↓	↑	NC	↑^	NC	NC
Anti-La	↓	↑	NC	↑^	NC	NC
RF	↑	↑	NC	NC	NC	NC
Cryoglobulins	↑	NC	NC	NC	NC	NC
Low complement	↑	NC	NC	NC	NC	NC
Lymphoma/death	↑/↑	NC/NC	NC/NS	NC/NS	NC/NS	NC/NS
Others	Genotype 1 more +SGB	↑ Gamaglobulins				
			SP-D Thr11/Thr 11 genotype	*CD5 haplotype rs2241002T-rs2229177C ^CD5 allele rs2241002C	＋CD6 haplotype rs17824933G-rs11230563C-rs12360861G λCD6rs11230563C ФCD6rs11230563T	^^ALCAM rs579565A ^ALCAM haplotype rs6437585C-rs579565G-rs1044243T

NC: no correlation, NE: not specified. PNS: peripheral nervous system, SGB: salivary gland biopsy. ILD: interstitial lung disease.

The Etiopathogenic Landscape of Sjögren's Syndrome

In the etiopathogenesis of SjS, a confluence of environmental determinants exerting influence upon genetically susceptible individuals has been posited as contributory to the condition's onset. Aberrant immunological responses—specifically involving T and B-lymphocytes—against autoantigens located in the exocrine glandular epithelium (such as Ro and La molecules) engender the synthesis of proinflammatory cytokines and chemokines. These molecules precipitate sustained inflammatory damage to the exocrine glands, culminating in attenuating their functional capacity (152-155). Epithelial cells in the context of SjS actively orchestrate and modulate localized autoimmune responses by mediating immune cells' recruitment, a ctivation, and differentiation. Concurrently, the inflammatory microenvironment and immune cells reciprocally act to either activate epithelial cells or modulate their longevity, thus engendering a self-perpetuating cycle of immune-epithelial cellular interactions that sustains the autoimmune manifestations observed in SjS (156-158).

While adaptive immunity—encompassing both T- and B-cell activations—has been implicated in the pathogenesis of SjS, the role of the innate immune response should not be marginalized. Innate immunity constitutes an immediate, biological defense mechanism that promptly incites inflammation and immune reactivity following pathogenic exposure. The principal cellular constituents of this defense strategy include dendritic cells, NK cells, epithelial cells, and macrophages, collectively designated as antigen-presenting cells. These cells serve as activators of the adaptive immune system and instigators of innate immune reactions and inflammation through specialized signaling pathways (159).

Owing to burgeoning insights into the intricate genetic underpinnings and the pivotal involvement of Type I and II interferons (IFNs) in systemic autoimmunity (160), contemporary research emphasizes the interplay between infectious agents and pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). This focus aligns with the burgeoning significance of gene-environment interactions in systemic autoimmune diseases, as TLR signaling implicates numerous genetically associated factors. Intriguingly, an infectious event may precipitate an autoimmune reaction well before clinical symptomatology, given that autoantibodies can be present for extended periods preceding the diagnostic confirmation of SjS (31, 84).

SGECs can also interface with innate immunity through TLR signaling, thus leading to the upregulation of immunologically competent molecules and synergizing innate and adaptive

immune responses (60,153). Additionally, TLR3 signaling has been shown to induce significant salivary gland hypofunction, SGECs apoptosis, and the upregulation of Ro/SSA and La/SSB autoantigens in SGECs, postulating its role in autoantigen presentation (60, 153, 161, 162). Further corroborating this notion is the SGECs' capability to mediate the exposure and production of intracellular proteins to the immune system through the secretion of autoantigen-rich vesicles, such as exosomes and apoptotic blebs from dying SGECs (60, 153). Experimental data from mouse models have buttressed the relevance of epithelial apoptosis in SjS, as evidenced by findings that the suppression of IκB-ζ expression in lacrimal epithelial cells led to increased apoptosis and subsequent development of SjS-like inflammatory lesions; this effect was reversible with caspase inhibitors, which inhibited apoptosis (163).

Regarding viral recognition by innate immunity, it is pertinent to discuss the role of collectins—C-type collagen-containing lectins. These soluble proteins function as pattern recognition receptors (PRRs) that discern pathogen-associated molecular patterns (PAMPs), including viral glycoproteins. Composed of trimerized units that oligomerize into higher-order structures, these innate immune proteins facilitate viral clearance via multiple mechanisms (103). Conversely, scavenger receptors (SRs) function as PRRs in immune defense and are exploited by various viruses for cellular entry (164).

The investigations constituting this doctoral thesis have been predicated on the aspiration to elucidate the pathogenic implications of factors regulating the innate immune system, both external (such as HCV) and internal (like genetic polymorphisms and innate immunity molecule levels), in patients diagnosed with SjS. Corroborating our initial hypothesis, we discerned divergent disease manifestations and immunological profiles based on the presence or absence of HCV and according to the presence or absence of specific gene variants—SP-D, CD5/CD6, and ALCAM—as well as serum levels of SP-D.

Viral Infections as Etiopathogenic Factors

Viral infections are significant etiological agents implicated in SjS, given that salivary gland tissue often harbors latent viral infections (67). The spectrum of implicated viruses is extensive, incorporating the EBV, which has been rigorously examined in the context of SjS (68). A notably elevated prevalence of serum HHV-6-specific antibodies have been documented in SjS patients as compared to control subjects (36% versus 10%) (69). Yet, this observation has not

been universally corroborated across studies (70, 71). Recent evidence has further elucidated immune responses (IgG) to EBV within this patient population (165). The analytical challenge in ascertaining the causative role of viral infections in SjS is augmented by the high prevalence of these viruses in the general population. Consequently, the evidence supporting a direct causative link between viral infections and the etiopathogenesis of SjS remains inconclusive.

Retroviruses, notably, have been recognized to infect immune system cells, leading to irregularities in immune modulation. Elevated serum levels of antibodies against HTLV-I and a heightened prevalence of salivary IgA-class anti-HTLV-I antibodies have been endemically reported in Japanese patients with SjS (72). Recent research also proves that HTLV-1 can directly infect SGECs (166).

For this doctoral thesis, our investigative focus is on the HCV as a potential etiopathogenic factor in SjS. HCV infection is a pervasive issue affecting approximately 184 million individuals globally. Its prevalence displays significant regional variation. For instance, the rates are notably high in Central Asia, Eastern Asia, and Middle East-North Africa, where more than 3.5% of the population is affected. Conversely, regions like Southeast Asia, the Andes, Central and Southern Latin America, Australia, the Caribbean, Oceania, and sub-Saharan Africa exhibit moderate prevalence rates (1.5–3.5%). The prevalence diverges substantially in Europe, ranging from below 1% in several Western European nations to up to 3% in Eastern Europe (167, 168).

Our investigation revealed an 8% prevalence of HCV infection in patients with SjS, according to the 2002 diagnostic criteria, which necessitate either anti-Ro/La positivity or a positive salivary gland biopsy. Studies from Mediterranean countries show a higher rate of chronic HCV infection among SjS patients—around 14%— (78). Additional research (82) has indicated a markedly increased prevalence of focal lymphocytic sialadenitis in salivary gland biopsies from patients with chronic HCV infection compared to those without HCV (57% vs. 5%). Other studies have observed prevalence variation, largely contingent upon geographic factors. Southern European studies report prevalence figures between 10% and 20% depending on the diagnostic tests employed (169).

In contrast, Scandinavian and U.S. studies report a virtually non-existent association, likely attributable to a lower regional prevalence of HCV. Our study may be subject to referral bias since it examined the largest SjS cohort from a singular center for HCV infection and revealed a prevalence rate substantially exceeding that in the broader Spanish population. Nonetheless, an

extensive Taiwanese study corroborates an elevated risk of SjS in HCV patients, with younger and male patients exhibiting higher susceptibility (170).

Genetic Susceptibility

The landscape of genetic susceptibility to SjS is intricate. Investigations into familial clustering indicate that approximately 35% of SjS patients possess relatives who also exhibit either SjS or a related autoimmune pathology, such as SLE or RA (62). An elucidative study involving 105 SjS patients with affected first-degree relatives has put forth compelling data, positing that the siblings of individuals with SjS manifest an elevated risk of SjS—approximately 662-fold (63). Like its autoimmune counterparts, SLE and RA, SjS is anticipated to be comparably complex, each boasting more than 100 recognized genetic associations. Nevertheless, the exploration of the genetic architecture underlying SjS remains conspicuously deficient.

Historically, the earliest genetic association studies in SjS were constrained by the focus on candidate gene approaches and needed to be more robust. Advances in methodology now employ genome-wide association studies (GWAS) that scrutinize associations with an extensive array of SNPs distributed across the entire genome. Within the scope of GWAS methodology, rigorous criteria for a statistically robust positive association are set at p-values less than 5×10^{-8}, while those ranging from 5×10^{-8} to 5×10^{-4} are categorized as suggestive. Transcending these genome-wide significance thresholds and corroborative data from independent cohorts is deemed indispensable for substantiating an "established" genetic association. Hence, identifying robust genetic associations is imperative for a comprehensive understanding of the complex genetic architecture of SjS.

In 2013, pioneering large-scale studies employing GWAS strategies in SjS were first published (64). These studies have definitively identified robust associations within the regions of human leukocyte antigens (HLA) Class I, III, and II, with particular emphasis on multiple effects concerning HLA-DR and DQ loci (64, 65). The alleles of HLA Class II have been discerned to be inextricably linked with autoantibody production in SjS, although not with other clinical features (65). Synergistic analyses integrating transcriptional data on gene expression levels with existing genetic data have unearthed associations categorized as expression quantitative trait loci (eQTLs) in five HLA Class I and II loci, namely A, C, DRB6, DPB1, and DQA1. These findings

insinuate that HLA risk alleles may influence peptide binding and antigen presentation and might also modulate expression levels of both class I and II molecules (171).

In 2012, Bolstad et al. unveiled the inaugural genome-wide significant association in a candidate gene investigation of the HLA class III locus (172). This seminal study discerned an association between a SNP located in the promoter region of the TNF gene and primary SjS. However, due to the pervasive linkage disequilibrium characterizing the entire HLA region, whether this association represents an independent genetic signal remains unresolved. A subsequent study in 2013 conducted by Lessard et al. engaged in the analysis of patients of European ancestry employing an array of large-scale genomic platforms (64). This work not only corroborated previously reported HLA associations but also identified genome-wide significant associations with six non-HLA loci (IRF5–TNPO3, STAT4, IL12A, FAM167A–BLK, CXCR5, and TNIP1). Concurrently, a Genome-Wide Association Study (GWAS) was published by Li et al., focusing on a Han Chinese cohort(173). This study substantiated associations between primary SjS and the loci GTF2IRD1–GTF2I and TNFAIP3—the latter having been prefigured in an antecedent candidate gene study and reaffirmed the involvement of STAT4 and the HLA region in primary SjS in Han Chinese populations. Validation of the GTF2IRD1–GTF2I association was subsequently provided by Song et al. in a 2016 GWAS study of Han Chinese female patients (174) and further elucidated by Zhao et al., who discovered a missense variant in NCF1 situated 62 kb from the implicated SNP rs11702632634 (175).

In 2017, Taylor et al. examined a heterogeneous patient population of European and Asian ancestries within Sjögren's International Collaborative Clinical Alliance (SICCA) initiative (176). This investigation illuminated conspicuous ancestry-specific variations in genetic associations with primary SjS, including those within the HLA locus, while reaffirming associations with the IRF5 and STAT4 genes. Complementarily, Carapito et al., in a separate 2017 study involving cohorts from France and the UK, reported an association between primary SjS and an allele of the non-conventional MHC-encoded class I gene, MICA33. Subsequent analyses confirmed that this association was independent of previously identified primary SjS-associated MHC class II signals (177).

In a divergent methodological strategy, Li et al. employed cis-expression quantitative trait locus (eQTL) analyses focusing on type I interferon-inducible transcripts. Their research unearthed multiple associations between primary SjS and a specific SNP in the OAS1 gene, which led to

altered gene-splicing patterns (178). Subsequently, a meta-analysis involving cohorts of European ancestry confirmed OAS1 as a locus susceptible to primary SjS. Adopting a similar candidate gene approach, Qu et al. ascertained a genome-wide significant association between primary SjS and the gene IKZF1 within a Han Chinese demographic (179). In a nuanced exploration using targeted sequencing, Thorlacius et al. substantiated and finessed the signal originating from the HLA locus, unmasking three distinct genetic signals in primary SjS patients who were positive for anti-SSA/Ro and anti-SSB/La autoantibodies (180). Notably, this study reported the most elevated odds ratio thus far—measured at 6.1—for a genetic association concerning patients who tested positive for either or both types of these specific autoantibodies.

In the most contemporary work, Khatri et al. executed a GWAS focusing on a patient cohort of European lineage (181). The study brought to light seven previously unidentified loci (NAB1, PTTG1–MIR146A, XKR6, MAPT–CRHR1, RPTOR–CHMP6–BAIAP2, TYK2, and SYNGR1). A subsequent meta-analysis, bolstered by additional ImmunoChip-derived data, revealed three more loci (CD247, PRDM1–ATG5, and TNFAIP3) attaining genome-wide significance in their association with primary SjS. Intriguingly, this investigation pioneered in calculating polygenic risk scores specific to primary SjS, achieving predictive values commensurate with those previously delineated for SLE and RA.

Seven non-HLA loci surpassing the genome-wide significance threshold have been delineated, implicating a broad array of innate and adaptive immune mechanisms. The specific immune pathways highlighted by these genetic associations as critical contributors to SjS encompass type 1 and type 2 interferon (IFN) signaling and responses (IRF5, IL12A, STAT4), NFκB signaling (TNIP1, TNFAIP3), lymphocyte trafficking (CXCR5), as well as the activation and differentiation of antibody-generating cells (BLK).

The mechanistic influence of these SjS-associated genetic variants on conventional biological processes remains largely elusive. Nevertheless, expression quantitative trait loci (eQTLs) have been ascertained for the IL12A, BLK, and TNIP1 genetic effects, which suggest that these associated variants modulate the transcriptional activity of the respective genes (64). Moreover, the specific immune cell subsets adversely affected by these variants are yet to be precisely delineated. While BLK expression is predominantly restricted to B-cells, transcripts of the other implicated genes have been identified in multiple cell subsets, including NK cells, monocytes, dendritic cells, and T and B-lymphocytes. Elucidating these genetic influences and the

ramifications of risk variants for immune system functionality holds vital implications for ascertaining the etiopathogenesis of SjS.

Limited studies explore the genetic variations of other primary SjS-related features, such as particular symptoms or comorbid conditions. However, in 2021, a meta-analysis involving primary SjS patients from Norwegian and Swedish cohorts discerned a genome-wide significant correlation between RTP4–MASP1 locus variants and fatigue symptoms among those with primary SjS (182). Although genome-wide significant associations remain unidentified concerning the primary SjS-associated comorbidity of lymphoma, multiple studies implicate the involvement of variants in TNFAIP3 in this specific comorbidity (61, 183, 184). In concert with additional genetic loci implicated in lymphoma pathogenesis in primary SjS but not achieving genome-wide significance, this gene has been comprehensively reviewed in separate literature (185, 186).

Genetic Polymorphisms and Innate Immune Molecules

The genetic research encompassed in this dissertation aims to scrutinize the frequency and clinical implications of genetic polymorphisms associated with the innate immune system's functionality, mainly focusing on surfactant and scavenger receptors.

Our findings revealed no statistically significant deviations in the prevalence of the Met11Thr polymorphisms within the SP-D gene when comparing patients with primary SjS to control subjects. This concurs with existing literature. Furthermore, the percentages paralleled those observed in healthy controls within preceding investigations conducted in European cohorts (Met11/Met11 35%, Thr11/Thr11 18%, Met11/Thr11 46%) (187).

In the context of patients diagnosed with primary SjS, our study did not discern any significant correlation between genetic polymorphisms and circulating SP-D levels. Other researchers have posited that relying solely on a single polymorphism to deduce serum levels may lead to underestimating the broader genetic influence, which might be more accurately represented by haplotype blocks (188). Sorensen et al. (109) suggested that serum SP-D levels are modulated not only by Met11Thr variations but also by additive genetic and environmental factors. Indeed, other investigations have reported a positive correlation between serum SP-D levels and variables such as male sex, age, smoking status, and physical activity (189). Additionally, SP-D oligomerization may be influenced by the Met11Thr polymorphism; previous studies have

intimated divergent roles in immune responses for SP-D multimers (anti-inflammatory) as opposed to SP-D trimers (pro-inflammatory) (190-192). In the context of primary SjS, it may be postulated that genetic polymorphisms exert a more localized influence on the etiopathogenic epithelial process. At the same time, serum SP-D levels might reflect an augmented systemic inflammatory response in patients exhibiting more significant clinical and immunological activity.

The pleiotropic function of SP-D is instrumental in preserving pulmonary homeostasis, facilitating the suppression of inflammation in unafflicted lungs while also instigating inflammation necessary for the active clearance of harmful pathogens, allergens, and apoptotic and necrotic cells (193). In systemic inflammation, serum SP-D appears to serve as an acute-phase reactant. Within systemic autoimmune diseases, elevated serum SP-D levels could either be a manifestation of nonspecific systemic inflammation—a byproduct of immune system activation—or could be intricately linked to the pathogenesis of the disease.

To summarize, the etiopathogenic relevance of SP-D in primary SjS appears to be predominantly modulated by peripheral serum levels rather than genetic polymorphisms.

We also executed case-control analyses to evaluate the modulatory impact of CD5, CD6, and CD166/ALCAM haplotypes on the risk of developing primary SjS. The data revealed that associations with primary SjS susceptibility were exclusively correlated with the CD166/ALCAM rs643785C-rs579565A-rs1044243C (CAC) and rs643785C-rs579565G-rs1044243T (CGT) haplotypes. These haplotypes exhibited a heightened prevalence in the case-cohort, implicating the rs579565A and rs1044243T alleles in the susceptibility to primary SjS.

When contrasted with controls, a marginal trend toward statistical significance was observed for the CD166/ALCAM rs579565A allele in primary SjS (q = 0.064). No differential expression in primary SjS versus controls was identified concerning the gene variants of CD5 and CD6.

Research focusing on patients with primary SjS needs to be more conspicuously expanded. The seminal clinical study in 2001 elucidated that individuals with primary SjS exhibited elevated concentrations of circulating sCD5 and sCD6 compared to controls. Furthermore, certain immunological features, namely hypocomplementaemia and cryoglobulinemia, correlate with heightened levels of these soluble scavenger receptors (194). Subsequent work by Papp et al. (195) revealed that CD5(+) B cells, but not their CD5(-) counterparts, manifested augmented levels of GrB and IL-21R in primary SjS. Moreover, elevated IL-21 expression in iNKT cells

was observed, thereby suggesting a pivotal role for enhanced IL-21R expression in CD19(+)CD5(+) B cells and IL-21 production by iNKT cells in primary SjS pathogenesis, potentially modulating CD19(+)CD5(+) B cell functions and elevating GrB production, thereby exerting a counter-regulatory impact on the disease. An additional investigation (149) employing a nuanced phenotypic analysis of B-lymphocytes in peripheral blood, bone marrow, and tonsils elucidated that CD6 expression is less closely aligned with CD5 expression than traditionally posited. Remarkably, CD6 was absent in transitional B cells but prevalent in mature and memory B cells. A noteworthy diminution in the proportion of CD6(+) B cells was observed in patients with primary SjS, contrary to those who have RA. This decrease did not emanate from the shedding of CD6 from B-cell membranes but rather from a reduction in memory B lymphocytes. This phenomenon might be attributable to CD6 facilitating the transmigration of CD27(+) memory B-cells into the salivary glands. Our findings are congruent with this hypothesis, as we observed elevated CD166 expression, a ligand for CD6, on the epithelial cells of the patient's salivary glands.

Influence on Demographic Profile and Glandular Damage

Notable epidemiological distinctions were discerned among HCV carriers; conversely, no such variances were evident about SP-D, CD5/CD6, or ALCAM gene variants. In our examination, patients with SjS concomitant with HCV (SjS-HCV) manifested a divergent epidemiological profile vis-a-vis those with primary SjS. Specifically, SjS-HCV was typified by a comparatively attenuated female-to-male ratio (5:1 as opposed to 14:1 in primary SjS cohorts) and an elevated median age at SjS diagnosis (62 years versus 56 years). Such observations deviate from the archetypal features of primary SjS, which customarily evinces an overt female predominance (10:1) and a younger median age at diagnosis (ranging between 30 and 50 years). The causative underpinnings for these disparate outcomes may be multifaceted, encompassing factors such as age at HCV diagnosis and the greater male susceptibility to HCV infection.

Sicca syndrome constitutes the principal clinical manifestation of SjS, thereby necessitating an in-depth evaluation of sicca symptoms and the diagnostic tests appraising the extent of glandular involvement (e.g., Schirmer test and parotid scintigraphy). Intriguingly, our data revealed that SP-D levels solely influenced glandular function; no significant correlations were discerned between HCV or CD5/CD6/ALCAM gene variants and the severity of symptoms or diagnostic

test outcomes for dryness. Elevated serum SP-D levels were correlated with severe dysfunction in salivary scintigraphy (grades III-IV), thus implicating SP-D as a pivotal mediator in glandular inflammation. Previous studies have corroborated the immunoreactivity of SP-D within the epithelial cells of both minor and major ducts of the parotid and lacrimal glands (107, 196). Regrettably, evaluations of SP-D SNPs yielded no significant correlations.

While the prevalence of sicca symptoms and the results of associated diagnostic tests were statistically equivalent in SjS-HCV and non-HCV patients, the clinical phenotype of SjS-HCV remained indistinguishable from that of primary SjS. The affinity of HCV for the salivary glands, termed "sialotropism", might elucidate its robust association with SjS. Experimental literature supports that HCV envelope proteins can recruit lymphocytes to the salivary glands, culminating in lymphocytic infiltrates analogous to those observed in primary SjS (focal sialadenitis) (82) (197,198). Pioneering research by De Vita et al. (199) augmented by two subsequent studies (200, 201), has validated the ability of HCV to infiltrate, infect, and replicate within salivary gland tissue in patients afflicted with sicca syndrome or SjS. Yet the specific mechanisms predisposing exocrine glands to HCV infection remain enigmatic. Intriguingly, genotype 1a of HCV was predominantly linked with positive salivary gland biopsies in contrast to other HCV genotypes, further complicating our understanding of the intricate relationships between HCV and glandular inflammation.

Influence on Extraglandular Involvement

The extent of systemic involvement in SjS is modulated by several factors, notably serum levels of SP-D and genetic variants in CD6 and ALCAM. Interestingly, SNPs in CD5 were found to be inconsequential for systemic manifestations of SjS. The most salient correlation was observed with serum SP-D levels, which exhibited a stronger association with disease phenotypes than Met11Thr polymorphisms. This underscores a tenuous correlation between SP-D polymorphisms and serum levels in patients with primary SjS. In these patients, the Met11Thr polymorphisms had clinical relevance only in their association with an elevated frequency of renal complications, particularly in those bearing the Thr11/Thr11 genotype. No additional correlations with other SP-D gene variants were observed in SjS, be they epidemiological, clinical, or immunological. These observations are congruent with existing literature on

autoimmune diseases, which also report a lackluster correlation between SP-D genotypes and clinical disease manifestation in RA (187, 202) and SLE (203).

Conversely, some studies have identified poor prognostic outcomes associated with genetic variants in patients with pulmonary diseases (204, 205). While the mechanisms governing SP-D synthesis remain enigmatic, they appear to be subject to genetic regulation. Among healthy subjects, research has demonstrated an association between the Thr11/Thr11 genotype and SP-D deficiency (109, 110).

Clinical implications of serum SP-D measurements are chiefly anchored on its correlation with chronic pulmonary diseases and malignancies (206-208). Several investigations have also assessed SP-D levels in patients with autoimmune and rheumatic systemic diseases, revealing decreased levels of SP-D in patients diagnosed with SLE (mean of 800 ng/ml) (203) and RA (mean range of 693–878 ng/ml) (187, 202), compared to healthy subjects who maintain an average level of > 900 ng/ml (187, 203). Our analysis corroborates these findings, reporting diminished levels of SP-D in patients with primary SjS (mean of 734 ng/ml), in alignment with levels observed in SLE and RA cohorts. Paradoxically, elevated SP-D levels have been documented in patients with SSc (209-2012), particularly those with pulmonary involvement.

Our study elucidates that despite the reduced mean SP-D levels in our primary SjS cohort, individuals with SjS-associated ILD exhibited elevated serum SP-D levels (> 1000 ng/ml), analogous to the high levels observed in patients with SSc-related ILD. This suggests that serum SP-D may serve as a viable soluble biomarker in clinical settings.

Consequently, elevated SP-D levels are expected in autoimmune diseases typified by frequent interstitial pulmonary involvement and, conversely, are decreased in conditions where pulmonary inflammatory involvement is less prevalent. This provides a compelling rationale for the correlation of SP-D levels with chronic pulmonary diseases, regardless of their etiological variations, given that SP-D is primarily synthesized in the pulmonary system.

SP-D is implicated in regulating pulmonary immune homeostasis, serving a dual function as an anti-inflammatory mediator under basal conditions and as an instigator of inflammation during pathogen elimination (102, 193, 213, 214). Elevations in serum SP-D levels have been discerned in correlation with disease activity in multiple pulmonary pathologies, including idiopathic ILD (215), HCV infections treated with interferon (216), and notably in SSc patients who manifest pulmonary complications (112,193, 203, 209, 210, 213).

Our study reveals an inverse association between SP-D levels and bronchiectasis, a recurrent pulmonary affliction in primary SjS (217). One plausible hypothesis for this divergence could be the frequent subclinical manifestation of bronchiectasis in primary SjS compared to ILD and the distinct etiopathogenic contributions of SP-D. The protein is substantially implicated in pulmonary tissue remodeling in animal models of lung fibrosis (218).

Moreover, our findings indicate elevated serum SP-D levels in primary SjS patients with concomitant renal involvement. Given that SP-D expression is not restricted to the lungs but is also detectable in extrapulmonary tissues, it raises the possibility of SP-D contributing to autoimmune-mediated tissue damage beyond the pulmonary system. Specifically, the presence of SP-D in renal glomeruli and tubules has been documented (219). Investigations by Hu et al. (220) and Xie et al. (221) substantiate that SP-D acts as an anti-inflammatory molecule in renal tubular epithelial cells and correlates with prognosis in chronic renal disease respectively. In conjunction with our data in primary SjS, these studies bolster the supposition that SP-D may have a functional role in renal pathology, mainly due to its potential proinflammatory actions in renal epithelial cells. Gardai et al. (213) elucidated that the inflammatory activity of SP-D could be either pro-inflammatory or anti-inflammatory, contingent upon the molecular orientation.

Serum SP-D levels could be a soluble indicator of systemic disease activity in primary SjS patients, particularly those with interstitial lung and renal involvement.

Furthermore, we observed that individuals carrying the CD6 rs11230563C allele were at increased risk for peripheral nervous system (PNS) ESSDAI activity, while the CD6 rs17824933G-rs11230563C-rs12360861G haplotype correlated with cutaneous ESSDAI activity. Prior research has implicated rs11230563C allele variants of the CD6 gene in increased susceptibility to MS and exacerbated Ps (128-132). These findings suggest a pivotal role for CD6 in the pathology of the nervous and integumentary systems, which are the same organs affected in our study.

Similarly, our results indicate an association between the CD166/ALCAM haplotype (rs6437585C-rs579565G-rs1044243T) and elevated PNS ESSDAI activity, aligning with observations made for CD6 rs17824933G and rs11230563C alleles in nervous system pathology. This substantiates the recognition of CD166/ALCAM as one of the most well-established ligands for CD6.

Influence on Immunological Profile

Our data elucidates that the autoantibody profile in SjS is substantially impacted by HCV, SP-D serum levels, and CD5 gene variants. Conversely, variants in the CD6 and ALCAM genes appear to exert minimal or negligible influence.

1. Anti-Ro/La Antibodies

We discerned distinct immunological patterns contingent upon the presence or absence of HCV infection. Among HCV-positive individuals, there was a diminished frequency of antinuclear antibodies (ANA) and Ro/La antibodies, yet an elevated frequency of RF, cryoglobulins, and hypocomplementemia relative to primary SjS patients. This observation carries multifaceted implications:

First, the prevalence of anti-Ro/La antibodies was markedly attenuated in patients afflicted with SjS concomitant with HCV compared to those lacking HCV infection. This immunological shift significantly impedes the fulfillment of the 2002/2016 classification criteria in HCV-positive patients, given that Ro/La positivity is indispensable in the absence of a confirmatory salivary gland outcome.

Second, our data revealed that more SjS-HCV patients harbor anti-La antibodies than those with anti-Ro antibodies, which sharply contrasts with findings in SjS patients devoid of HCV infection.

Third, we noted that approximately one-fourth of Ro/La+ SjS-HCV patients possessed exclusive anti-La antibodies, an immunological nuance infrequently documented in primary SjS (<5%). This lends credence to the hypothesis that HCV co-infection markedly perturbs the autoimmune response against the human La protein, a perturbation likely related to the virus's utilization of human ribonucleoproteins.

The human La protein has been identified as an indispensable host factor for HCV RNA translation and replication (223). Translation of HCV, a critical facet of its viral replication, is facilitated by an internal ribosome entry site, with La ribonucleoprotein serving as a potent modulator of this replication process (224). We investigated the conjecture that anti-La antibody presence could confer protection against chronic HCV infection. Regrettably, our findings do not corroborate a significant protective role for serum anti-La antibodies against HCV chronicity in SjS patients. Of the seven SjS patients exhibiting resolved HCV infection (indicated by positive HCV-IgG and negative HCV-RNA), none manifested anti-La antibodies. Additionally, no salient

differences emerged in the epidemiological, clinical, and immunological profile of anti-La-positive patients depending upon HCV status.

Previous research implicates lymphotropic viruses in the genesis of autoimmune diseases. Antigenic epitopes on the 48 KD La/SS-B protein demonstrate molecular homology to specific viral domains in EBV, HHV-6, and HIV-1 (225). Such antigenic cross-reactivity, mediated by viral-induced translocation of self-antigens to the cell surface, may foster the production of autoantibodies. Recent data indicate that 13% of a sizable Spanish cohort of SjS patients harbored HCV infection. This subset had a notable preponderance of anti-La over anti-Ro antibodies, contrary to their HCV-negative counterparts.

Regarding the impact of genetic polymorphisms on innate immune molecules, our research demonstrates that elevated levels of SP-D substantially alter the immunological profile in SjS. Specifically, our data indicate that high serum SP-D concentrations are more robustly correlated with the presence of positive RF, anti-Ro, and anti-La antibodies, as well as markers of B-cell hyperactivity such as hypergammaglobulinemia and elevated IgG levels. Conversely, there is a weaker association with acute-phase reactant proteins. Given that no other studies have investigated the role of SP-D in SjS, our preliminary findings necessitate validation through larger, more comprehensive investigations. Serum SP-D levels could serve as a valuable marker for tracking disease activity.

Furthermore, our study reveals a notable association between the CD5 rs2241002C allele and the minor CD5 rs2241002T-rs2229177C haplotype with specific manifestations of SjS. These genetic variations were previously implicated in a more severe form of SLE, particularly lupus nephritis (168). In the context of SjS, carriers of these CD5 variants showed increased anti-Ro/anti-La antibody positivity, anemia, and thrombocytopenia. This may indicate the hyperactivity of autoantibody-producing B-cells, most plausibly CD5+ B1a cells.

Additionally, our findings establish an association between the CD166/ALCAM haplotype (rs6437585C-rs579565G-rs1044243T) and heightened incidences of ANA positivity and hematological cytopenias. These results bolster the evidence supporting the harmful effects of CD6 rs17824933G and rs11230563C alleles, as well as the CD166/ALCAM rs1044243T allele, in exacerbating select analytical and clinical parameters of SjS. Intriguingly, our haplotypic analyses revealed that CD166/ALCAM rs6437585C-rs579565A-rs1044243C and rs6437585C-rs579565G-rs1044243T haplotypes are associated with an elevated susceptibility to primary SjS.

This lends credence to a role for the minor rs579565A and rs1044243T alleles in primary SjS vulnerability, echoing the earlier age of MS diagnosis that has been attributed to the rs579565A allele (145).

2. Cryoglobulins

Mixed cryoglobulins are documented in approximately two-thirds of patients with SjS-HCV, representing a ninefold increase in prevalence compared to patients with primary SjS. These cryoglobulins serve a pivotal function in the overarching immunological profile of these patients and exhibit a strong correlation with positive RF, monoclonal gammopathy, and diminished levels of C3 and C4 complement components. The incidence of these markers, whether in isolation or combination, was notably elevated compared to patients devoid of HCV infection. Indeed, our analysis revealed statistically significant disparities in serum monoclonal expression—both in frequency and heterogeneity—between SjS patients contingent upon the presence or absence of HCV infection. Among patients with SjS-HCV, the prevalence of circulating monoclonal immunoglobulins (mIgs) was threefold more significant than in those without HCV. Specifically, mIgMκ, intimately linked with mixed cryoglobulinemia, emerged as the most detected circulating mIg.

Conversely, in HCV-negative patients, mIgGk was the predominant circulating monoclonal band. Moreover, we discerned that SjS-HCV patients presenting with monoclonal gammopathy exhibited a more constricted monoclonal expression profile, predominantly confined to either mIgMk or mIgG, in contrast to HCV-negative patients, who manifested a diverse range of monoclonal heavy and light chains. These observations imply that HCV may substantially influence the clonal selection of B-cells.

The primary etiological factor for mixed cryoglobulinemia (MC) types II and III is chronic HCV infection, accounting for 70-90% of cases (226-231). In comprehensive prospective studies, MC presence is identified in approximately 40-60% of patients with HCV infection, although merely 5-10% eventually manifest cryoglobulinemic vasculitis (232, 233). Asymptomatic carriers of cryoglobulinemia do not appear to follow a divergent clinical trajectory compared to their cryoglobulin-negative counterparts. Nevertheless, patients afflicted with chronic hepatitis C who also possess circulating cryoglobulins have been reported to experience elevated rates of cirrhosis and amplified fibrosis scores after controlling for variables such as age, gender, and

duration of infection when contrasted with those lacking circulating cryoglobulins (168, 234). The presence or absence of advanced fibrosis was not correlated with the type and concentration of cryoglobulins.

As for the underlying mechanisms of cryoglobulin, pathogenicity is most comprehensively elucidated in the context of HCV-associated cryoglobulinemia. The CD81 receptor, ubiquitously expressed in B lymphocytes and hepatocytes, enables HCV to infect both cell types (152, 226, 235). Active HCV replication has been demonstrated in CD19-positive B cells. HCV-RNA and HCV core and NS3 proteins can be identified exclusively in CD19-positive peripheral blood mononuclear cells but not in their CD19-negative counterparts (236). Additionally, HCV replication has been documented in other cells, such as monocytes, peripheral dendritic cells, and macrophages (237, 238). Chronic HCV-induced stimulation prompts lymphocytes to produce an array of autoantibodies facilitated by the virus-induced reduction in cellular activation thresholds. This profuse autoantibody production engenders a multitude of immune manifestations correlated with HCV infection, cumulatively termed "HCV syndrome" (225, 239-242). Consequently, HCV syndrome may encompass a broader range of clinical manifestations, extending beyond the hallmark features of mixed cryoglobulinemia to include autoimmune thyroiditis, sicca syndrome, thrombocytopenia, haemolytic anaemia, autoimmune diabetes, and pulmonary fibrosis (225, 235, 243).

An HCV-induced gene translocation inhibits B cell apoptosis, leading to oligoclonal monotypic lymphoproliferation (244). In the context of HCV-related MC, lymphoid infiltrations comprising cells expressing either oligo- or monoclonal rheumatoid factor are identifiable in multiple organs, including portal tracts, spleen, and bone marrow. As such, MC establishes an intersection between traditional autoimmune diseases and hematologic malignancies—specifically, B-cell lymphomas. The persistent activation of B-cells by viral antigens, coupled with the heightened expression of genes related to lymphomagenesis (notably activation-induced cytidine deaminase, essential for somatic hypermutation (245), fosters a transition from polyclonal to monoclonal B-cell expansion. These cumulative interactions eventually precipitate a lymphoproliferative disorder that may progress to B-cell non-Hodgkin's lymphoma. Strong correlations have been identified among diverse hematologic malignancies, including large B-cell lymphoma, marginal zone lymphoma, and lymphoplasmacytic lymphoma (246).

A chronically stimulated viral milieu—commonly associated with HCV—represents the most extensively studied pathogenetic mechanism for MC. This model is predicated on: i. the production of IgM rheumatoid factor and resultant cryoprecipitable immune complexes; ii. aberrant kinetics leading to tissue deposition of these immune complexes, compounded by an inefficient clearance by monocytes/macrophages (which exacerbates glomerular damage); and iii. an insidious, quiescent lymphoproliferative disorder.

Circulating immune complexes composed of HCV, anti-HCV polyclonal IgG, and monoclonal IgM are perpetuated by a stable B-cell clone, initiated by chronic HCV infection. These cryoprecipitate complexes evade the erythrocyte transport system due to clonally specific IgM (239), directly influencing hepatic and splenic macrophages that are ill-equipped to process them owing to lysosomal enzyme biogenesis abnormalities (239). Similarly, possibly due to HCV infection of phagocytic cells, a corresponding dysfunction appears likely in circulating monocytes. These cells have been observed to contain cryoglobulins under electron microscopy evaluation of tissue specimens (247) while demonstrating an incapacity to process phagocytosed immune material (248).

The precise role of monocytes/macrophages in triggering cryoglobulinemic nephritis remains elusive. Whether the migration of phagocytes to the glomerulus—a foundational event in renal involvement—exacerbates or mitigates damage through cryoglobulin removal is a subject of ongoing debate. Specific in vitro models (248) indicate that the sequestration of cryoglobulins by monocytes/macrophages signifies inefficient cryoglobulin clearance, potentially perpetuating glomerular injury. This notion is corroborated by a murine model of cryoglobulinemic membranoproliferative glomerulonephritis (249), where macrophage elimination afforded protection against mesangial expansion and collagen accumulation without impacting cryoglobulin removal. Such data support the hypothesis that macrophage migration to the glomerulus is pivotal in advancing renal damage (249). This is more in line with the exacerbation of injury following immune complex deposition rather than an adaptive cryoglobulin clearance mechanism. The alteration in lysosomal enzymes, such as pro-cathepsin D (248), and danger-associated molecular patterns (DAMPs) emitted from injured resident cells (249) might undermine macrophage's innate ability to clear immune complexes via Fc gamma receptors. On the other hand, mesangial expansion and activation might be propagated by extracellular activation of released pro-cathepsin D (248) or proinflammatory cytokines emanating from

DAMPs-activated macrophages (249). Moreover, clonally restricted IgM demonstrates a strong affinity for glomerular matrix components, such as fibronectin, suggesting the potential for an "in situ" binding mechanism (250).

Cryoglobulinemia is characterized by serum immunoglobulins that reversibly precipitate into a gel-like structure when exposed to temperatures below 37°C and redissolve upon re-warming. According to Brouet's classification (251), this condition is categorized into three subtypes based on immunoglobulin composition. Type I cryoglobulinemia consists of single monoclonal immunoglobulins, while Types II and III are denoted as MC, encompassing both IgG and IgM components. Types II and III cryoglobulins function as immune complexes of polyclonal IgGs— autoantibodies—and either mono- or polyclonal IgMs, respectively. In this context, IgMs are the corresponding autoantibodies with RF activity, defined as the ability to bind another antibody. Employing more sensitive methodologies like immunoblotting, two-dimensional polyacrylamide gel electrophoresis, or advanced immunofixation techniques frequently elucidates the micro-heterogeneous composition of type II mixed cryoglobulins. The presence of oligoclonal IgM or a combination of polyclonal and monoclonal IgM may be identified. Such a serological subset, termed type II–III MC, could represent a transitional stage between type III and type II MC (252). Variability in this phenomenon is primarily attributed to factors such as population selection, lead time biases, and the lack of standardized clinical assessment. Additionally, laboratory tests for MC are susceptible to false-negative results (232, 233). Moreover, the duration of cryoglobulinemia has been observed to correlate with the longevity of HCV infection, enduring twice as long in HCV-positive patients manifesting cryoglobulinemia (232).

The fundamental mechanism underlying cryoglobulinemia is aberrant autoantibody synthesis by B cells, coupled with B-cell proliferation. Numerous etiological diseases may potentiate this by perturbing normal B-cell function. Type I cryoglobulins are invariably associated with B-cell lymphoproliferative disorders, including multiple myeloma, Waldenström macroglobulinemia, chronic lymphocytic leukemia, B-cell non-Hodgkin lymphoma, and hairy cell leukemia. Conversely, mixed cryoglobulinemias (Types II and III) are linked with systemic autoimmune diseases, lymphoproliferative disorders, and chronic infections. HCV infection constitutes approximately 80% of the cases of mixed cryoglobulinemic vasculitis. Autoimmune diseases implicated include primary SjS, SLE, and RA. When MC manifests without a well-defined underlying disorder, the condition is termed "essential MC."

MC in primary SjS correlates with extra glandular manifestations, elevated risk of B-cell lymphoma, and diminished survival rates. Similarly, approximately 10% of patients with SLE and RA exhibit MC, albeit with generally lower cryocrit levels than in SjS and less frequent clinical manifestations (253-257). Technically, "cryoglobulinemia" refers solely to the serum presence of cryoglobulins. However, this term is often expanded to describe a systemic inflammatory syndrome characterized by small-to-medium vessel vasculitis instigated by cryoglobulin-containing immune complexes. Terminology such as "cryoglobulinemic syndrome" and "cryoglobulinemic vasculitis" delineate the clinically apparent disorder and asymptomatic cryoglobulin presence. Distinctions are made between cryoglobulinemic syndrome, characterized by symptoms like purpura, arthralgia, and weakness, and more severe vasculitic manifestations, including skin ulcers, peripheral nervous system involvement (e.g., pure sensory axonopathy, mononeuritis multiplex, or most frequently distal sensory or sensory-motor polyneuropathy), and renal complications (259). Predominantly, type II and type II–III mixed cryoglobulins, and less frequently type III, can result in a specific clinical landscape categorized under systemic vasculitides affecting small and occasionally medium-sized vessels. Most of these cases correlate with HCV infection (38). In the context of type II MC, they may be linked to low-grade proliferative B-cell lymphomas (38, 260).

Linking Cryoglobulins, HCV, Lymphoma, and SjS

Our findings indicate that patients with SjS coexisting with the SjS-HCV exhibit a higher incidence of adverse clinical outcomes. These include hematological neoplasia (9% vs. 4%, P=0.04), general neoplasia (20% vs. 8%, P<0.001), and mortality (33% vs. 8%, P<0.001) in comparison to patients devoid of HCV infection. Notably, no significant associations were discerned between SP-D levels or gene variants of SP-D, CD5/CD6, and ALCAM/CD166.

The lymphotropic characteristics of HCV contribute to the production of cryoglobulins and the onset of lymphoma (261). A heightened prevalence of hematological neoplasia was observed in patients with SjS-HCV compared to their counterparts without HCV. Mechanistically, lymphomagenesis in HCV-infected individuals may be initiated through the viral agent's chronic activation of polyclonal B-cells (262). Moreover, categorizing HCV quasispecies in blood mononuclear cells (263) could lead to subsequent B-cell clonal expansion and oncogenic

mutations (264, 265). These mechanisms closely resemble the etiopathogenic pathways underlying lymphoma development in primary SjS (262, 266, 267).

Chronic HCV infection is intricately linked to lymphoproliferative and autoimmune disorders (268, 269). A higher prevalence of lymphoproliferative disorders in HCV-infected individuals has been well-documented (270,271). Additionally, HCV's particular tropism for various extrahepatic cell types establishes a compelling connection between HCV infection and autoimmune disease pathogenesis (198, 272-275). HCV's sialotropism might account for its specific association with SjS, while its lymphotropism links the virus to cryoglobulin synthesis and lymphoma genesis. This dual extrahepatic tropism of HCV heightens the likelihood of autoimmune and lymphoproliferative disease manifestation in patients with chronic HCV infection. Recent research corroborates the similarity in etiopathogenic underpinnings of lymphoma development in both SjS and HCV cohorts (263, 276).

Primary SjS is the systemic autoimmune disease most associated with lymphoma risk (277). Our data elucidate that the conjunction of HCV and SjS substantially amplifies the risk relative to primary SjS alone. SjS and chronic HCV infection are underpinned by hyperactive B-cell activity, predisposing them to monoclonal B-cell selection (278). As such, SjS-HCV coexistence may pose one of the highest risks for overt B-cell lymphoma across all systemic autoimmune disorders. This elevated risk profile unsurprisingly corresponds with a poor prognosis and elevated mortality rates. Recent evidence has shown that compared to HCV-cryoglobulinemia patients, those with primary SjS-cryoglobulinemia exhibited more frequent lymphadenopathy, type II IgMk cryoglobulins, and lymphoma (OR=6.12, 95% CI: 2.7-14.4). Conversely, these patients manifested less frequently C4 hypocomplementemia and peripheral neuropathy (279).

Ramos-Casals et al. (280) Delineated patients' clinical and immunological features with SjS-HCV, who later developed B-cell lymphoma. Contrasted with data procured from the SjS-HCV Registry involving patients without lymphoma (281), our cohort of SjS-HCV lymphoma patients demonstrated distinct clinical and immunological profiles, which should be interpreted within the context of the divergent study designs and recruitment criteria. Clinically, a heightened prevalence of parotid gland enlargement (32% vs. 12%) and vasculitic manifestations— encompassing cutaneous vasculitis (56% vs. 18%) and peripheral neuropathy (44% vs. 16%)— was observed in SjS-HCV patients with B-cell lymphoma. Notably, parotid enlargement is a clinical indicator strongly suggestive of lymphoma in primary SjS cases (264, 282). The elevated

frequency of this symptom in our patient group reaffirms its strong correlation with lymphoma development in SjS-HCV cohorts. The remarkable frequency of vasculitic features and cryoglobulins aligns with their association with cryoglobulinemia (227, 282, 283) and lymphoma. It suggests their potential as prognostic indicators for lymphoma development (262).

Immunologically, most of our SjS-HCV patients with lymphoma were RF positive, exhibiting almost double the prevalence compared to those without lymphoma (281). RF secretion by polyclonally activated B-cells is implicated in lymphoma genesis in both SjS and HCV contexts but is unrelated to other RF+ pathologies such as rheumatoid arthritis (284). Intriguingly, our data posits RF as a novel predictive factor for lymphoma in SjS-HCV patients despite it having not been previously established in primary SjS or HCV-only populations.

The preponderance of MALT lymphoma in our SjS-HCV cohort stood in stark contrast to the high-grade lymphomas frequently observed in non-selected HCV patients. This pattern underscores the influential role of SjS in the lymphomagenesis of SjS-HCV patients, echoing previous research (277, 285). Our study indicates that MALT lymphoma in SjS-HCV cases also carries prognostic implications; patients with this subtype had significantly better survival outcomes than those with other lymphoma types.

Lastly, our findings demonstrated a predilection for extranodal B-cell lymphoma localization in SjS-HCV patients, particularly in the exocrine glands, liver, and stomach, accounting for 14 of the 25 cases studied. This suggests that HCV-induced infection and replication in these organs lead to localized B-cell activation and RF production, thereby setting the stage for neoplastic transformation. Such an anatomical pattern further substantiates the etiopathogenic affinity between SjS-HCV lymphomas and those directly associated with SjS compared to lymphomas related solely to HCV infection (282).

CONCLUSIONS

1. HCV-SjS patients are distinguised by having a specific phenotype of disease which clearly differs from those with primary SjS:

    They are predominantly male and are diagnosed at a higher age.

    They have a lower frequency of anti-Ro/La antibodies.

    They have an abnormal predominant presence of anti-La among anti-Ro antibodies

    They have a higher frequency of cryoglobulinemic-related immunological markers (RF, hypocomplementemia, mIgs)

    They have a higher prevalence of lymphoma and a higher prevalence of mortality.

2. Primary SjS patients with high serum levels of SP-D are characterized by having:

    More systemic activity (glandular involvement, ILD, renal involvement).

    A higher prevalence of positive antibodies Ro/La.

3. The specific association between SP-D levels and pulmonary and renal involvements may have pathophysiological implications.

4. There is no association between serum SP-D levels and the Met11Thr SP-D SNPs.

5. Primary SjS susceptibility was exclusively correlated with:

    CD166/ALCAM rs643785C-rs579565A-rs1044243C (CAC) and rs643785C-rs579565G-rs1044243T (CGT) haplotypes.

    These haplotypes implicate the rs579565A and rs1044243T alleles in the susceptibility to primary SjS.

6. Primary SjS patients with CD5 SNPs have:

    Higher prevalence of anti-Ro/La antibodies (rs2241002C allele polymorphism)

    Higher prevalence of cytopenias (rs2241002T-rs2229177C haplotype)

7. Primary SjS patients with CD6 SNPs have:

    Higher prevalence of skin involvement (rs17824933G-rs11230563C-rs12360861G)

    Higher prevalence of PNS involvement (rs11230563C)

    Higher prevalence of neutropenia (CD6rs11230563T)

8. Primary SjS with CD166/ALCAM rs6437585C-rs579565G-rs1044243T haplotype have:

    Higher prevalence of PNS involvement

    Higher prevalence of cytopenias and ANAs antibodies

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APENDIX

Original article

Cryoglobulinaemic vasculitis at diagnosis predicts mortality in primary Sjögren syndrome: analysis of 515 patients

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Abstract

Objective. To evaluate the fulfilment of classification criteria for cryoglobulinaemic vasculitis (CV) at diagnosis in a large cohort of patients with primary SS and their correlation with poor outcomes.

Methods. We included 515 consecutive patients tested for serum cryoglobulins who fulfilled the 2002 classification criteria for primary SS. CV classification criteria and serum cryoglobulins at diagnosis were assessed as predictors of death and lymphoma using Cox proportional-hazards regression analysis adjusted for age and gender.

Results. Positive serum cryoglobulins were detected in 65 (12%) patients, of whom 21 (32%) fulfilled CV classification criteria. Compared with patients positive for cryoglobulins who did not fulfil CV criteria, patients with CV had a higher frequency of type II cryoglobulinaemia (86% *vs* 43%, P = 0.04), a higher mean cryocrit level (6.58% *vs* 1.25%, P < 0.001) and a higher cumulated mean EULAR-SS disease activity index score (35.3 *vs* 16.2, P < 0.001). After a mean follow-up of 110 months, 45 (9%) patients developed B-cell lymphoma and 33 (6%) died. Compared with patients without cryoglobulins, patients with cryoglobulins who fulfilled [hazard ratio (HR) = 7.47, 95% CI: 3.38, 16.53] and did not fulfil (HR = 2.56, 95% CI: 1.03, 6.35) CV criteria both showed a higher risk of B-cell lymphoma in the univariate analysis, but not in the multivariate models. Compared with patients without cryoglobulins, patients with CV had a higher risk of death in both the univariate (HR = 11.68, 95% CI: 4.44, 30.74) and multivariate (HR = 4.36, 95% CI: 1.32, 14.47) models.

Conclusion. Patients with primary SS who fulfilled criteria for cryoglobulinaemic vasculitis at diagnosis are at higher risk of death.

Key words: Sjögren syndrome, cryoglobulinaemia, vasculitis, mortality, ESSDAI

Rheumatology key messages

- Four per cent of patients with primary SS fulfilled at diagnosis the 2014 classification criteria for cryoglobulinaemic vasculitis.
- Presence of cryoglobulinaemic vasculitis at diagnosis of primary SS is independently associated with higher systemic activity and mortality.
- Patients with primary SS should be evaluated at diagnosis for systemic activity (EULAR-SS disease activity index) and for cryoglobulinaemic vasculitis.

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Introduction

SS is a systemic autoimmune disease that principally affects women between the fourth and sixth decades of life [1]. The clinical spectrum of SS extends from dryness of the main mucosal surfaces to systemic involvement, which may be the presenting manifestation or appear after the disease is diagnosed [2]. While sicca symptoms significantly worsen the quality of life, systemic features clearly mark the disease prognosis. The development of the EULAR-SS disease activity index (ESSDAI) [3] by the EULAR task force on SS has represented a step forward in the characterization of systemic SS, including specific organ-by-organ definitions and allowing the homogeneous evaluation of systemic features in large series of patients [4–6]. The identification at diagnosis of systemic markers prospectively associated with a poor prognosis could play a significant role in identifying patients with primary SS requiring closer follow-up and early treatment.

Cryoglobulins are immunoglobulins that precipitate *in vitro* at temperatures below 37 °C and redissolve after rewarming. The term cryoglobulinaemia refers to the presence of cryoglobulins in serum, while cryoglobulinaemic disease or cryoglobulinaemic vasculitis (CV) are terms used to describe symptomatic patients [7]. In primary SS, some CV-related features have individually been associated with a poor prognosis, including cutaneous purpura, low C4 levels, monoclonal gammopathy and serum cryoglobulins [8]. All these features are now included in the recently published preliminary classification criteria for CV [9, 10], which may allow the evaluation of the whole spectrum of CV as a prognostic factor in primary SS. The aim of this study was to evaluate the fulfilment of CV classification criteria at diagnosis in a large cohort of Southern European patients with primary SS and their correlation with poor outcomes.

Patients and methods

Patients

Serum cryoglobulins were consecutively tested at diagnosis in 515 consecutive patients (371 from the Hospital Clinic, Barcelona and 144 from the University of Udine) who were diagnosed with primary SS according to the 2002 American–European criteria until December 2014 [11]. Patients with other possible causes of sicca syndrome, chronic viral infections and concomitant systemic autoimmune diseases were excluded. Diagnostic tests for SS (ocular tests, parotid scintigraphy and salivary gland biopsy) were made according to the European Community Study Group recommendations [11]. Clinical and laboratory data were collected and computerized according to a standard protocol [4]. The study design (retrospective, non-interventional) conformed to current Spanish ethical standards. The study was approved by the Clinical Research Ethics Committees of the two hospitals, Hospital Clinic, Barcelona, Spain and Azienda Ospedaliero Universitaria S. Maria della Misericordia, University of Udine, Udine, Italy and complied with the ethical standards of the Helsinki Declaration.

Serum cryoglobulins were measured after centrifugation according to previously reported standard techniques [12, 13]. Blood samples were obtained and maintained at 37ºC for 30 min before separation. Serum was prepared by centrifuging at 37ºC for 10 min at 2500 revolutions per minute. Fresh, centrifuged serum was incubated at 4ºC for 7 days after collection, and examined for cryoprecipitation. Cryoglobulins were further analysed by immunofixation when >5% of cryoprecipitate was available. Serum monoclonal immunoglobulins were analysed by immunofixation electrophoresis on agarose gels with specific antisera to IgG, IgM, IgA, κ and λ chains at diagnosis and every year during the follow-up.

All patients were followed prospectively after diagnosis with regular hospital visits at 6- to 12-month intervals. The individual observation time for all patients was from the first determination of cryoglobulins until the last hospital visit, transfer out, diagnosis of neoplasia or death. The outcomes measured were development of B cell haematological neoplasia and death. Haematological neoplasia was classified according to the revised fourth version of the 2008 WHO classification for tumors of haematopoietic and lymphoid tissues [14].

Definition of variables

The date of disease diagnosis was defined as the date when the physician responsible for the patient's follow-up confirmed fulfilment of the 2002 criteria [11]. Systemic involvement at diagnosis was defined according to the ESSDAI [3], which evaluates 12 domains or organ systems. Each domain is divided into three to four levels according to the degree of activity and scored as 0 (no activity), 1 (low activity), 2 (moderate activity) or 3 (high activity). The ESSDAI score at diagnosis was retrospectively calculated by examination of medical records in order to collect disease activity before the date of SS diagnosis [15].

The CV classification criteria were retrospectively evaluated, including the following three items [9, 10]: a validated questionnaire for CV; data on the pattern of organ involvement (present and past), including constitutional symptoms, articular involvement, vascular involvement and neurological involvement; and laboratory tests (positive RF, reduced C4 level, presence of serum monoclonal component). Patients fulfilled CV criteria when they had serum cryoglobulins (by at least two repeated tests) and fulfilled at least two of the three items at the time of diagnosis or during the first year of follow-up.

Statistical analysis

Descriptive data are presented as means and (S.D.) or median and interquartile range (IQR) for continuous variables and numbers and percentages (%) for categorical variables. Three groups of patients were defined according to the presence of serum cryoglobulins (negative *vs* positive) and fulfilment of criteria in patients with positive serum cryoglobulins (yes *vs* no).

Table 1 Fulfilment of items and sub-items included in the CV criteria

| Variables | Cryoglobulins (+)[a] | | Cryoglobulins (−)[a] | |
	CV (+)Group 1 (n = 21)	CV (−)Group 2 (n = 44)	Group 3 (n = 450)	P-value[b]
Item 1[c]	20 (95.2)	7 (15.9)	36 (8)	<0.001
Item 2 (at least 3)	18 (85.7)	5 (11.4)	31 (6.9)	<0.001
Constitutional symptoms	16 (76.2)	13 (29.5)	137 (30.4)	<0.001
Fatigue	12 (57.1)	9 (20.5)	94 (20.9)	0.001
Low grade fever 37–37.9 °C	9 (42.9)	8 (18.2)	47 (10.4)	<0.001
Fever, >38 °C	1 (4.8)	1 (2.3)	23 (5.1)	0.791
FM	2 (9.5)	0 (0)	34 (7.6)	0.102
Articular involvement	16 (76.2)	27 (61.4)	208 (46.2)	0.005
Arthralgias	14 (66.7)	21 (47.7)	175 (38.9)	0.025
Arthritis	8 (38.1)	9 (20.5)	54 (12)	0.003
Vascular involvement	21 (100)	11 (25)	66 (14.7)	<0.001
Purpura	21 (100)	6 (13.6)	33 (7.3)	<0.001
Skin ulcers	6 (28.6)	0 (0)	3 (0.7)	<0.001
Necrotizing vasculitis	3 (14.3)	0 (0)	3 (0.7)	0.002
Hyperviscosity syndrome	0	0	0	1.000
RP	6 (28.6)	4 (9.1)	31 (6.9)	0.006
Neurological involvement	15 (71.4)	6 (13.6)	59 (13.1)	<0.001
Peripheral neuropathy	15 (71.4)	5 (11.4)	40 (8.9)	<0.001
Cranial nerve involvement	1 (4.8)	3 (6.8)	15 (3.3)	0.292
Vasculitic CNS involvement	0 (0)	0 (0)	6 (1.3)	1.000
Item 3 (at least two)	20 (95.2)	17 (38.6)	91 (20.2)	<0.001
Reduced serum C4	20 (95.2)	11 (25)	45/448 (10)	<0.001
Positive RF	18 (85.7)	34/43 (79.1)	265/444 (59.7)	0.003
Positive monoclonal comp	13/19 (68.4)	11/38 (28.9)	74/329 (22.5)	<0.001

The values are represented as number (%). [a]Groups of patients: patients who fulfilled CV criteria (group 1), patients positive for serum cryoglobulins who did not fulfil CV criteria (group 2) and patients negative for serum cryoglobulins (group 3). [b]P-values were calculated using Fisher's exact test. [c]For Item definitions see supplementary Table S4, available at *Rheumatology* Online.

Quantitative variables were compared between the three groups using the non-parametric Kruskal–Wallis rank sum test, because normality and equality of variance could not be assumed. Fisher's exact test was used to compare categorical outcomes. Dunn's test with Bonferroni adjustment was used for multiple pairwise comparisons. The association between items and sub-items included in the CV criteria and the outcomes (lymphoma and death) was evaluated using univariate analysis. The fulfilment of CV classification criteria and the presence of serum cryoglobulins were assessed as predictors of death and lymphoma using univariate Cox proportional-hazards regression analysis adjusted for age at diagnosis and gender. The total number of objective CV features included in items 2 and 3 (0, 1, 2, 3, 4 or more) was also assessed as a predictor of death and lymphoma. Multivariate Cox proportional hazards regression analysis was performed including the fulfilment of the CV classification criteria (yes/no), the presence of serum cryoglobulins (yes/no) and the total number of objective CV features (0, 1, 2, 3, 4 or more) adjusted for age at diagnosis and gender in order to establish which of these categorical variables were independently associated with death and/or lymphoma. A sensitivity analysis was performed including patients with only one positive determination of serum cryoglobulins among the group of positive criteria. The hazard ratios (HRs) and their 95% CIs obtained in the adjusted regression analysis were calculated. All significance tests were two-tailed and values of $P < 0.05$ were considered significant. All analyses were conducted using the R version 3.0.3 for Windows statistical software package.

Results

Fulfilment of CV criteria

The cohort included 479 (93%) females and 36 (7%) males, with a mean age at diagnosis of 52.9 years. Positive serum cryoglobulins were detected in 65 (12%) patients, of whom 21 (32%) fulfilled the 2014 classification criteria for CV, 4 (6%) tested positive in the only available determination available and 40 (62%) had ⩾2 determinations but only one positive determination. Table 1 summarizes the fulfilment of the items and sub-items included in the CV criteria in the three groups. Differences were statistically significant ($P < 0.001$) for all variables except for those with the lowest prevalence (fever >38ºC, hyperviscosity syndrome, FM, cranial nerve involvement and CNS vasculitis).

Table 2 Main features at diagnosis of patients with primary SS

| | Cryoglobulins (+)[a] | | Cryoglobulins (–)[a] | |
	CV (+) Group 1 (n = 21)	CV (–) Group 2 (n = 44)	Group 3 (n = 450)	P-value[b]
Variables				
Gender, male, n (%)	2 (9.5)	3 (6.8)	31 (6.9)	0.773
Age at present, mean (S.D.), years	62.2 (11.5)	62.1 (14)	62.1 (16)	0.994
Age at diagnosis, mean (S.D.), years	50.9 (13.9)	51.3 (13.9)	53.2 (15.3)	0.529
Dry mouth, n (%)	19 (90.5)	42 (95.5)	431 (95.8)	0.376
Dry eye, n (%)	20 (95.2)	41 (93.2)	426 (94.7)	0.895
Altered ocular tests, n (%)	21 (100)	41/42 (97.6)	382/424 (90.1)	0.106
Altered parotid scintigraphy, n/N (%)	13/15 (86.7)	26/27 (96.3)	263/317 (83)	0.192
Positive salivary gland biopsy, n/N (%)	16/16 (100)	30/32 (93.8)	190/202 (94.1)	0.763
Anaemia, Hb < 110 g/l, n/N (%)	8/20 (40)	10 (22.7)	70/449 (15.6)	0.014
Leukopenia, <4000/mm^3, n (%)	12 (57.1)	22 (50)	118 (26.2)	<0.001
Thrombocytopenia, <150000/mm^3, n (%)	3 (14.3)	4 (9.1)	35 (7.8)	0.440
Neutropenia, <1500/mm^3, n (%)	1 (4.8)	14/43 (32.6)	75 (16.7)	0.013
Lymphopenia, <1000/mm^3, n (%)	10 (47.6)	9/43 (20.9)	49 (10.9)	<0.001
ANA+, n/N (%)	17/19 (89.5)	42 (95.5)	387/446 (86.8)	0.270
RF+, n/N (%)	15 (71.4%)	27/43 (62.8)	224/444 (50.5)	0.062
Anti-Ro/SS-A+, n (%)	17 (81)	37 (84.1)	321/449 (71.5)	0.144
Anti-La/SS-B+, n (%)	12 (57.1)	28 (63.6)	225/449 (50.1)	0.211
Monoclonal gammopathy, n/N (%)	13/19 (68.4)	11/38 (28.9)	74/329 (22.5)	<0.001
Low C3 levels, <0.82 g/l, n/N (%)	11/20 (55)	10/43 (23.3)	44/447 (9.8)	<0.001
Low C4 levels, <0.11 g/l	20 (95.2%)	11 (25%)	45/449 (10%)	<0.001
ESSDAI domains (activity), n (%)				
Constitutional domain	16 (76.2)	12 (27.3)	131 (29.1)	<0.001
Lymphadenopathy domain	13 (61.9)	17 (38.6)	72 (16)	<0.001
Glandular domain	13 (61.9)	19 (43.2)	130 (28.9)	0.002
Articular domain	16 (76.2)	27 (61.4)	207 (46)	0.005
Cutaneous domain	21 (100)	13 (29.5)	93 (20.7)	<0.001
Pulmonary domain	5 (23.8)	10 (22.7)	48 (10.7)	0.016
Renal domain	4 (19)	3 (6.8)	11 (2.4)	0.002
Muscular domain	0 (0)	0 (0)	7 (1.6)	1.000
Peripheral nervous system domain	15 (71.4)	6 (13.6)	48 (10.7)	<0.001
CNS domain	1 (4.8)	2 (4.5)	20 (4.4)	1.000
Haematological domain	18 (85.7)	33 (75)	293 (65.1)	0.076
Biological domain	21 (100)	44 (100)	246 (54.7)	<0.001
Total ESSDAI scores, mean (S.D.)				
At diagnosis	12 (11.4)	8.8 (8.2)	5.7 (6.3)	<0.001[c,d]
Cumulative until the last visit	35.3 (14)	16.2 (10.6)	10.5 (9.1)	<0.001[c,d,e]

[a]Groups of patients: patients who fulfilled CV criteria (group 1), patients positive for serum cryoglobulins who did not fulfil CV criteria (group 2) and patients negative for serum cryoglobulins (group 3). [b]P-values were calculated using the Kruskal–Wallis rank sum test for quantitative variables and Fisher's exact test for categorical variables. [c]Statistically significant (P < 0.05) differences between groups 1 and 3 (Dunn's test of multiple comparisons with Bonferroni adjustment). [d]Statistically significant (P < 0.05) differences between groups 2 and 3 (Dunn's test of multiple comparisons with Bonferroni adjustment). [e]Statistically significant (P < 0.05) differences between groups 1 and 2 (Dunn's test of multiple comparisons with Bonferroni adjustment). n, N: number.

SS characterization

Table 2 summarizes the main features at diagnosis of the 21 patients who fulfilled CV criteria, the 44 with serum positive cryoglobulins who did not fulfil the criteria and the 450 patients who were negative for serum cryoglobulins. Compared with patients positive for serum cryoglobulins who did not fulfil CV criteria, patients with CV had a higher frequency of type II cryoglobulinaemia (86% *vs* 43%, P = 0.04) and a higher mean cryocrit level (6.58 *vs* 1.25%, P < 0.001). With respect to the ESSDAI domains, patients

with CV had a higher global cumulated mean ESSDAI score (35.3 *vs* 16.2, P < 0.001) and a higher frequency of systemic activity in the constitutional (76 *vs* 27%, P < 0.001), cutaneous (100 *vs* 29%, P < 0.001) and peripheral nervous system (71% *vs* 14%, P < 0.001) domains. With respect to laboratory parameters, patients with CV had a higher frequency of monoclonal gammopathy (68 *vs* 29%, P = 0.009), low C3 levels (55 *vs* 23%, P = 0.021), low C4 levels (95 *vs* 25%, P < 0.001) and lymphopenia (47.6% *vs* 20.9%, P = 0.042) but a lower frequency of neutropenia (4.8 *vs* 32.6%, P = 0.014). The statistical differences were greater when

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Fɪɢ. 1 Kaplan–Meier curves according to fulfilment of CV criteria for overall death and B cell lymphoma
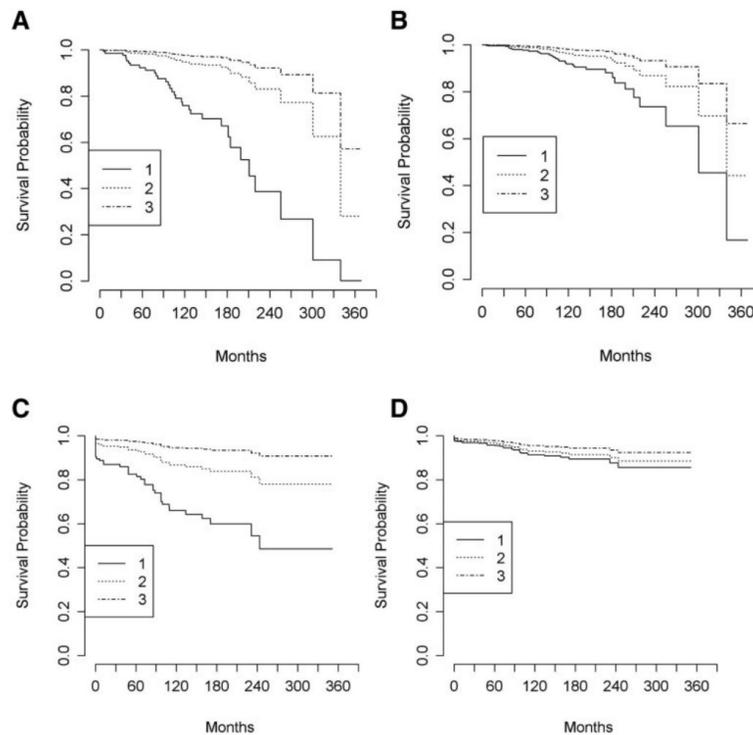
Fulfilment of CV criteria (1 = fulfilment, 2 = no fulfilment with cryo+, 3 = no cryoglobulins). (**A**, **B**) Univariate and multivariate models for death adjusted for age at diagnosis and gender. (**C**, **D**) Univariate and multivariate models for B-cell lymphoma adjusted for age at diagnosis and gender.

CV patients were compared with patients without cryoglobulinaemia (Table 2).

Outcomes

The median follow-up was 128.4 months (IQR 64.6–174.0) in patients who fulfilled CV criteria, 123.8 months (IQR 50.5–194.4) in patients with serum positive cryoglobulins who did not fulfil the criteria, and 98.9 months (IQR 42.1–154.2) in patients who were negative for serum cryoglobulins. There were no significant differences in the median follow-up between the three groups (P = 0.059). Thirty-three (6%) patients died after a mean follow-up of 110.5 months (range 0–368 months), and 45 (9%) developed B cell lymphoma. Figure 1 shows the corresponding Kaplan–Meier curves, both unadjusted and adjusted for age at diagnosis and gender, for survival and the development of lymphoma in the three groups. Compared with patients without cryoglobulins, patients who fulfilled CV criteria had a higher risk of death in the univariate

(HR = 11.68, 95% CI: 4.44, 30.74) and multivariate (HR = 4.36, 95% CI: 1.32, 14.47) models (Table 3). Patients with cryoglobulins who fulfilled (HR = 7.47, 95% CI: 3.38, 16.53) and did not fulfil (HR = 2.56, 95% CI = 1.03, 6.35) CV criteria both showed a higher risk of B cell lymphoma in the univariate analysis, but not in the multivariate models. The sensitivity analysis (supplementary Table S1, available at *Rheumatology* Online) including the four patients with only one positive determination of cryoglobulins showed similar results. With respect to the total number of objective CV features included in items 2 and 3, patients with ⩾4 features had a higher risk of death in the univariate analysis, but not in the multivariate models, and a higher risk of B cell lymphoma in both the univariate (HR = 20.00, 95% CI: 2.62, 152.86) and multivariate (HR =14.65, 95% CI: 1.82, 117.77) models (Table 3 and Fig. 2). An additional analysis (supplementary Table S2, available at *Rheumatology* Online) showed that patients negative for cryoglobulins were protected against lymphoma (HR = 0.39) compared with patients with CV.

TABLE 3 Fulfilment of criteria and number of clinical/immunological CV criteria as predictors of death or lymphoma

Variables	n (%)	Death (n = 33)			Lymphoma[a] (n = 37)		
		N	Univariate HRs (95% CI)[b]	Multivariate HRs (95% CI)[c]	N	Univariate HRs (95% CI)[b]	Multivariate HRs (95% CI)[c]
Fulfilment of criteria[d]							
Group 1	21 (4.1)	7	**11.68 (4.44, 30.74)**	**4.36 (1.32, 14.47)**	9	**7.47 (3.38, 16.53)**	1.95 (0.78, 4.87)
Group 2	44 (8.5)	4	2.28 (0.75, 6.90)	2.00 (0.63, 6.30)	6	**2.56 (1.03, 6.35)**	1.55 (0.61, 3.95)
Group 3	450 (87.4)	22	Ref	Ref	22	Ref	Ref
No. of criteria							
0	68 (13.2)	5	Ref	Ref	1	Ref	Ref
1	123 (23.9)	4	0.76 (0.20, 2.89)	0.76 (0.20, 2.94)	1	0.56 (0.03, 8.91)	0.55 (0.03, 8.75)
2	149 (28.9)	7	0.96 (0.29, 3.15)	0.99 (0.30, 3.29)	7	2.96 (0.36, 24.26)	2.85 (0.35, 23.35)
3	97 (18.8)	5	1.09 (0.30, 3.94)	1.00 (0.26, 3.70)	7	4.64 (0.57, 37.97)	4.32 (0.53, 35.58)
⩾4	78 (15.2)	12	**6.02 (1.87, 19.36)**	3.42 (0.89, 13.14)	21	**20.00 (2.62, 152.86)**	**14.65 (1.82, 117.77)**

Bold indicates statistically significant (P < 0.05) factors associated with death and/or lymphoma. [a]Excluded from the analysis eight patients in whom lymphoma was diagnosed previously. [b]Univariate Cox proportional-hazards regression analysis adjusted for age at diagnosis and gender. [c]Multivariate Cox proportional-hazards regression analysis adjusted for age at diagnosis and gender. [d]Groups of patients: patients who fulfilled CV criteria (group 1), patients positive for serum cryoglobulins who did not fulfil CV criteria (group 2) and patients negative for serum cryoglobulins (group 3). n, N: number; Ref: reference level.

Supplementary Table S3, available at *Rheumatology Online*, summarizes the results of a univariate analysis that evaluated the individual association between the different items and sub-items included in the CV criteria and the main outcomes (lymphoma and death). All three main items and the majority of sub-items were associated with lymphoma and/or death, except for articular involvement, which was a protective factor. Low grade fever, purpura (both subjective and objective), skin ulcers, reduced serum C4 levels and serum monoclonal component were associated with the two outcomes.

Discussion

Although primary SS is often considered a chronic disease principally characterized by the triad of dryness, fatigue and pain, systemic involvement has increasingly been recognized as an important component of the disease spectrum. Three multicentre studies, including more than 2500 European patients, have recently confirmed that primary SS is, undeniably, a systemic autoimmune disease [4–6], with systemic activity (defined as an ESSDAI score ⩾ 1) present at diagnosis in 70–80% of patients. Systemic disease plays a key role in the prognosis of primary SS, especially in patients with serum cryoglobulins [16].

Although cryoglobulins may be detected in a wide range of systemic autoimmune diseases [7], cryoglobulinaemia is most closely related to SS [17]. Cryoglobulins are detected in up to 10% of patients with SLE and RA, but cryocrit values are generally lower and the clinical manifestations of CV vasculitis much more infrequent compared with primary SS patients [7]. In 1998 we were the first to report the close association between cutaneous leucocytoclastic vasculitis, hypocomplementaemia, chronic HCV infection and cryoglobulins in patients with

SS [12], and in 2007 we were also the first to prospectively demonstrate the key prognostic role of cryoglobulins in patients with primary SS without HCV infection [18]. Previous studies had reported the prognostic role of some features closely related to cryoglobulinaemic syndrome. In 2002 Ioannidis *et al*. [19] were the first to propose a prognostic classification of patients with primary SS according to the presence of palpable purpura and/or low C4 levels (types I and II patients), and in 2004 Theander *et al*. [20] found that the strongest predictors of a poor outcome were low C3/C4 levels at diagnosis. Recent studies in larger series of patients have confirmed the key role of some of the main CV-related features, including purpura [4, 21], low C4 [4, 6, 22], monoclonal gammopathy [23] and serum cryoglobulins [4, 6, 22] in the prognosis. In a recent study [15], we found that the main baseline features associated with a higher risk of death were systemic activity at diagnosis, cytopenias, monoclonal gammopathy, hypocomplementaemia and cryoglobulins. Cryoglobulins may play a central role in the poor prognosis of patients with SS for three main reasons: the most severe extraglandular manifestations of SS are often related to cryoglobulinaemic disease, cryoglobulins are closely associated with other immunological prognostic markers (hypocomplementaemia, monoclonal band), and patients with cryoglobulinaemia have a higher risk of B cell lymphoma.

However, until now, all studies that have searched for prognostic factors in primary SS have analysed these CV-related factors one by one. The present study is the first to evaluate the complete spectrum of clinical and immunological features now integrated in the definition of CV according to the internationally accepted classification criteria [7]. Many patients with cryoglobulinaemia remain asymptomatic, and the percentage of patients with circulating cryoglobulins who develop vasculitic symptoms

Fɪɢ. 2 Kaplan–Meier curves according to the number of the items included in the CV criteria for overall death and B cell lymphoma
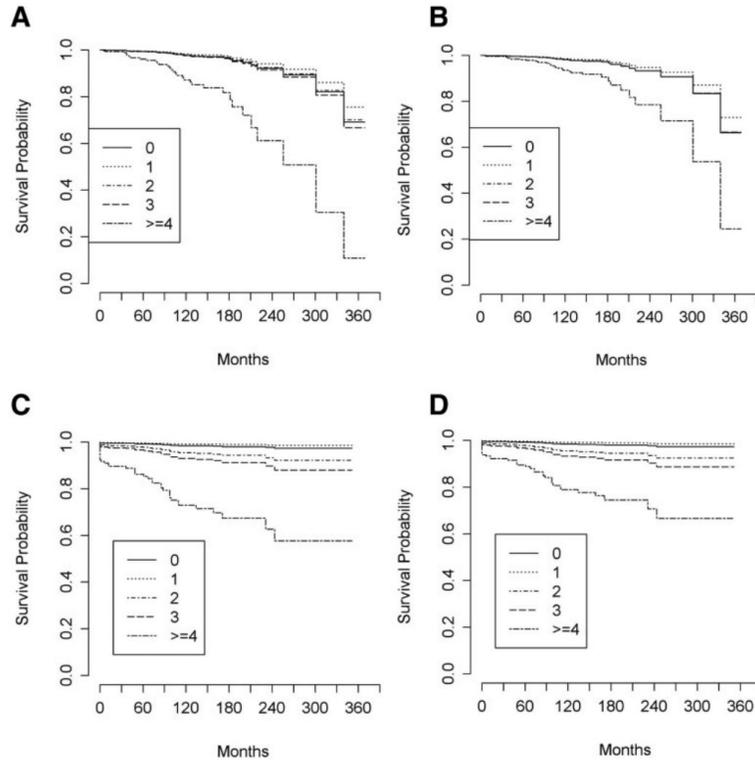
Number of the main items included in the CV criteria (0, 1, 2, 3, 4 or more). (A, B) Univariate and multivariate models for death adjusted for age at diagnosis and gender. (C, D) Univariate and multivariate models for B-cell lymphoma adjusted for age at diagnosis and gender.

varies between 2 and 50% [7]. In our patients with primary SS and positive cryoglobulins, the percentage of symptomatic cryoglobulinaemic syndrome, defined by fulfilment of item 2, was 35% (86% in those fulfilling CV criteria compared with 11% in those who did not). Only 7% of patients without cryoglobulinaemia fulfilled item 2: this shows that, although there may be some clinical overlap between systemic SS and CV, the need for fulfilment of at least three of the four clinical domains included in the item 2 of the CV classification criteria (constitutional, articular, vascular and neurological involvement) permits good discrimination between the two diseases.

Systemic vasculitis (mainly related to cryoglobulins) is one of the main autoimmune causes of death in patients with primary SS [8, 15]. However, the clinical expression of vasculitis depends on the location of the vessels affected, with the skin being the organ predominantly involved. In primary SS, the clinical expression of CV ranges from benign disease (arthralgias and uncomplicated cutaneous purpura) to life-threatening systemic vasculitis [8]. There is a close association between vasculitis and SS, and this is clearly reflected in the ESSDAI index: the ESSDAI classifies cutaneous vasculitic activity as moderate or high according to the cutaneous extension ($<$ or $>$18% of the body surface area involved, respectively) and the presence of ulcers (high activity), while extracutaneous vasculitis, including renal and neuropathic cryoglobulinaemic involvement, is included in the corresponding renal and peripheral nerve system ESSDAI domains. We have confirmed the strong link between CV and the ESSDAI in our patients: patients with CV had the highest percentages of activity in all organ domains except two (muscular and CNS, which are those with the lowest prevalence), and mean scores were 2-fold higher

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at diagnosis and 3-fold higher at the end of follow-up compared with the scores of patients negative for cryoglobulins.

This study is the first to link worse survival of primary SS patients with CV at diagnosis, defined according to the international classification criteria [9,10], when compared with both patients negative for cryoglobulins and patients with cryoglobulinaemia who did not fulfil CV criteria. This was also valid for patients with only one positive determination of cryoglobulins when the other clinical and immunological criteria were fulfilled. We also show that the more clinical/immunological CV-related markers (⩾4) primary SS patients have, the worse the survival, although this was not confirmed in the age–gender adjusted model due to the significant influence of these two variables on mortality (older patients and men had a worse prognosis, data not shown). CV at diagnosis of primary SS, defined according to the classification criteria, and not according to serum cryoglobulins, is a major prognostic marker.

We also found an association between cryoglobulinaemia and lymphoma, which was stronger in patients with CV (HR = 7.47) than in those without (HR = 2.56), although the differences were not significant in the multivariate model adjusted by age and gender. In addition, patients negative for cryoglobulins are protected against lymphoma (HR = 0.39) compared with patients with CV. However, and in contrast to the survival analysis, we also found that the more clinical and immunological CV-related markers (⩾4) the primary SS patient has, the higher the risk of lymphoma (in both the univariate and multivariate analyses). Our results highlight the key role of cryoglobulinaemia in lymphoma development in primary SS patients [24–27]. In patients with primary SS, cryoglobulinaemia should be differentiated from hypergammaglobulinaemic purpura, which is also associated with cutaneous purpura but is related to polyclonal B cell hyperactivity, whereas CV is a monoclonal-driven vasculitis that should be considered as a pre-lymphomatous condition [22]. The application of the CV criteria may help physicians differentiate between CV and hypergammaglobulinaemic purpura when a patient with primary SS presents with cutaneous purpura at diagnosis.

Not all features included in the CV criteria had the same weight of association with a poor outcome. The three main items (subjective symptoms, organ-specific involvement and laboratory parameters) and the majority of sub-items were associated with lymphoma and/or death, except for articular involvement, which was a protective factor. Low grade fever, purpura (both subjective and objective), skin ulcers, reduced serum C4 levels and serum monoclonal component were associated with the two outcomes. These results suggest that some features have a stronger influence on the prognosis than others. A limitation of our study is its retrospective design, and the results should be confirmed in future studies with an age–gender matched case-control design.

The presence of CV at the diagnosis of primary SS is independently associated with mortality, and is closely linked with higher baseline systemic activity measured by the ESSDAI. Patients with primary SS should be tested at diagnosis for cryoglobulins, RF, C3/C4 complement factors and serum immunoelectrophoresis, and should be evaluated both for systemic activity (ESSDAI) and for vasculitis (fulfilment of CV criteria). Patients with primary SS and concomitant CV at diagnosis should be closely followed and treated early due to the high risk of adverse outcomes.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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How are we treating our systemic patients with primary Sjögren syndrome? Analysis of 1120 patients [☆]

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ARTICLE INFO

ABSTRACT

Objective: To describe how systemic disease is treated in a large cohort of Spanish patients with primary Sjögren syndrome (pSS) in daily practice, focusing on the adequacy of therapies for the level of systemic activity measured by ESSDAI score.

Patients and methods: By December 2014, our database included 1120 consecutive patients who fulfilled the 2002 classification criteria for SS. Therapeutic schedules were classified into 4 categories: no systemic therapies, hydroxychloroquine (HCQ) and/or low dose glucocorticoids (GCS) (<20 mg/day), high dose GCS (>20 mg/day) and use of second-line therapies (immunosuppressive agents, intravenous immunoglobulins [IVIG] and/or rituximab [RTX]).

Results: There were 1048 (94%) women and 72 (6%) men, with a mean age at diagnosis of 54 years. The main drug-based therapeutic approaches for systemic pSS during follow-up were HCQ in 282 (25%) patients, GCS in 475 (42%, at doses >20 mg/day in 255—23%), immunosuppressive agents in 148 (13%), IVIG in 25 (2%) and RTX in 35 (3%) patients. HCQ was associated with a lower risk of death (adjusted HR of 0.57, 95% 0.34–0.95). We classified 16 (7%) of the 255 patients treated with >20 mg GCS and 21/148 (14%) treated with immunosuppressive agents as patients inadequately treated, mainly associated with articular involvement of low/moderate activity.

Conclusion: The management of pSS should be organ-specific, using low dose GCS in patients with moderate systemic activity, limiting the use of high dose GCS and second-line therapies to refractory or potentially severe scenarios. The use of systemic therapies for dryness, chronic pain or fatigue is not warranted.

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1. Introduction

Sjögren syndrome (SS) is a systemic autoimmune disease that mainly affects the exocrine glands, causing dryness of the main mucosal surface, such as the mouth, eyes, nose, pharynx, larynx and vagina [1]. However, the clinical spectrum of SS extends from dryness to systemic involvement (extraglandular manifestations) and may include a large number of manifestations. The disease overwhelmingly affects middle-aged women, but may also affect children, men and the elderly. SS may be a severe disease with excess mortality, mainly related to systemic involvement and lymphoma, and is expressed in many guises, depending on the specific epidemiologic, clinical, or immunologic features [2].

The therapeutic management of SS is principally centered on control of the main symptoms, sicca features, using substitutive and oral muscarinic agents [3]. However, systemic involvement clearly marks the disease prognosis. The development of the EULAR-SS disease activity index (ESSDAI) [4] by the EULAR task force on SS is a step forward in the evaluation of patients with systemic Sjögren, who should receive a closer follow-up and more robust therapeutic management [5]. As a rule, the management of systemic Sjögren should be organ-specific, with glucocorticoids and immunosuppressive/biological agents limited to potentially-severe scenarios. However, a systematic review highlighted the limited evidence available for the drugs most frequently used in primary SS and the difficulties of offering solid therapeutic recommendations [6]. In this scenario, information about how these complex patients are treated in a real life setting may be very useful.

The aim of this study was to describe how systemic Sjögren is treated in a large cohort of Spanish patients with primary SS in daily practice, focusing on the adequacy of drug therapies for the level of systemic activity measured by the ESSDAI score.

2. Patients and methods

2.1. Patients

The GEAS-SS Study Group was formed in 2005 with the aim of collecting a large series of Spanish patients with primary SS, and included 21 Spanish centers with substantial experience in the management of patients with systemic autoimmune diseases. Both incident and prevalent cases were included; for incident cases, the diagnosis of primary SS was made during the first study visit after January 2005, while for the prevalent cases, the diagnosis was established before January 2005. By December 2014, the database included 1120 consecutive patients (686 prevalent cases) who fulfilled the 2002 classification criteria for pSS [7]. Exclusion criteria were chronic HCV/HIV infection, previous lymphoproliferative processes and associated systemic autoimmune diseases. Diagnostic tests for SS (ocular tests, parotid scintigraphy and salivary gland biopsy) were performed according to the European Community Study Group recommendations [7].

2.2. Definition of variables

The date of disease diagnosis was defined as the date when the physician responsible for the patient's follow-up confirmed fulfillment of the 2002 criteria [7]. Systemic involvement was defined according to the ESSDAI [4], which evaluates 12 domains or organ systems. Each domain is divided into 3–4 levels according to the degree of activity and scored as 0 (no activity), 1 (low activity), 2 (moderate activity) or 3 (high activity). The ESSDAI score at diagnosis was retrospectively calculated by examination of medical records in order to collect disease activity before the date of SS diagnosis. Disease activity states (DAS) were defined according to the baseline ESSDAI score (low DAS for an ESSDAI <4, moderate DAS for an ESSDAI between 5 and 13, and high DAS for an ESSDAI >13) [8]. Therapeutic schedules were classified into 4 categories: no systemic therapies, HCQ and/or low dose of GCS

(<20 mg/day), high dose of GCS (>20 mg/day) and use of second-line therapies (immunosuppressive agents, IVIG and/or RTX). According to the systemic definitions included in the ESSDAI, we defined the use of GCS (>20 mg/day) in patients with an ESSDAI score < 4 (equivalent to moderate arthritis, defined as 1–5 affected joints) and the use of second-line therapies in patients with an ESSDAI score <6 (equivalent to severe arthritis, moderate vasculitis or severe cytopenia) as inadequate therapeutic regimens.

2.3. Statistical analysis

Descriptive data are presented as means and standard deviation (SD) for continuous variables and numbers and percentages (%) for categorical variables. Systemic therapies were categorized as never or ever used during the follow-up, and included the use of HCQ, oral GCS (higher or lower than 20 mg/day), immunosuppressive agents (cyclophosphamide, azathioprine, mycophenolate and methotrexate), IVIG and RTX. The clinical and immunological characteristics and presence/level of activity recorded in the ESSDAI organ domains were assessed at diagnosis. To compare the main baseline features of patients according to the use or not of systemic therapies t-test or chi-square tests were used. Logistic multivariate regression model was constructed to analyze independent factors associated with the use or not of systemic therapies. Variables with a p-value < 0.1 in the univariate analysis were included in the model and stepwise model selection by Akaike information criterion (AIC) was used. Cox proportional-hazards regression analysis allowed adjustment for age at diagnosis, gender and level of ESSDAI activity as confounders, in order to establish independent systemic therapy variables associated with the outcomes evaluated such as lymphoma, death or a combination of both. The hazard ratios (HR) and their 95% confidence intervals (CI) obtained in the adjusted regression analysis were calculated. All significance tests were 2-tailed and values of p < 0.05 were considered significant. All analyses were conducted using the R version 3.0.3 for Windows statistical software package.

3. Results

3.1. Baseline characterization

Baseline characteristics are summarized in Table 1. The cohort consisted of 1120 patients, including 1048 (94%) women and 72 (6%) men (female: male ratio, 15:1), with a mean age at diagnosis of 54.45 ± 15.21 years (range, 14–90). At diagnosis, 1065 (95%) patients presented dry mouth, 1062 (95%) dry eye, 955/1042 (92%) had altered ocular diagnostic tests (Schirmer's test and/or corneal stainings), 731/841 (87%) altered parotid scintigraphy and 485/557 (87%) a salivary gland biopsy showing focal lymphocytic infiltration. The main immunologic features at diagnosis were ANA >1/80 in 1004/1118 (90%) patients, anti-Ro/SS-A in 823/1116 (74%), RF in 566/1083 (52%), anti-La/SS-B in 508/1113 (46%), low C4 levels in 127/1057 (12%), cryoglobulinemia in 81/652 (12%) patients, low C3 levels in 105/1058 (10%) and monoclonal gammopathy in 88/879 (10%). The mean total ESSDAI score at diagnosis was 5.91 ± 6.77.

3.2. Systemic therapeutic approaches and baseline features

The main drug-based therapeutic approaches for systemic Sjögren ever used during the follow-up were HCQ in 282 (25%) patients, GCS in 475 (42%, used at doses > 20 mg/day in 255—23%), immunosuppressive agents in 148 (13%), IVIG in 25 (2%) and RTX in 35 (3%) patients. According to therapeutic schedules, 634 (57%) patients were untreated, 183 (16%) were treated with HCQ and/or low dose of GCS, 132 (12%) with high doses of GCS and the remaining 170 (15%) with the addition of immunosuppressive agents, IVIG and/or rituximab. Table 2 compares the main baseline features of patients according to the use or not of systemic therapies. The use of systemic therapies

Table 1
Baseline characteristics of a Spanish cohort of 1120 patients with primary SS.

Variables at diagnosis	N = 1120 (%)
Gender (male)	72 (6.4)
Age (mean ± SD)	54.45 ± 15.21
Dry mouth	1065 (95.1)
Dry eyes	1062 (94.8)
Altered ocular tests	955/1042 (91.7)
Altered parotid scintigraphy	731/841 (86.9)
Positive salivary gland biopsy	485/557 (87.1)
Anemia (Hb <110 g/l)	202/117 (18.0)
Leukopenia (<4000/mm^3)	213/1118 (19.1)
Thrombocytopenia (<150,000/ mm^3)	85/1117 (7.6)
Neutropenia (<1500/mm^3)	124/1117 (11.1)
Lymphopenia (<1000/mm^3)	138/1116 (12.4)
Antinuclear antibodies >1/80	1004/1118 (89.8)
Rheumatoid factor	566/1083 (52.3)
Anti-Ro/SS-A	823/1116 (73.7)
Anti-La/SS-B	508/1113 (45.6)
Monoclonal gammopathy	88/879 (10.0)
Cryoglobulins	81/652 (12.4)
Low C3 levels (<0.82 g/l)	105/1058 (9.9)
Low C4 levels (<0.11 g/l)	127/1057 (12.0)
Baseline ESSDAI (mean ± SD)	5.91 ± 6.77

was more frequent in males ($p = 0.036$) and was associated with the presence at diagnosis of anemia ($p < 0.001$), thrombocytopenia ($p = 0.001$), neutropenia ($p = 0.002$), rheumatoid factor ($p < 0.001$), anti-Ro/SS-A ($p = 0.033$), monoclonal band ($p = 0.002$), cryoglobulins ($p = 0.003$), low C3 ($p = 0.003$) and low C4 ($p = 0.014$). The ESSDAI score at diagnosis was higher in patients who received systemic therapy than in those who did not (8.12 vs. 4.20, $p < 0.001$). Multivariate analysis identified neutropenia, rheumatoid factor and the ESSDAI score as independent variables. Fig. 1 summarizes the use of the different therapeutic approaches according to disease activity states (DAS).

3.3. Systemic therapies and outcomes

After a mean follow-up of 111 months, 32 (3%) patients developed B-cell lymphoma and 121 (11%) patients died. The cumulated mean total ESSDAI score at the end of follow-up was 12.03. Table 3

Table 2
Baseline characteristics of a Spanish cohort of 1120 patients with primary SS according to the use of systemic therapies.

Variables at diagnosis	No systemic therapy N = 635 (%)	Systemic therapy N = 485 (%)	Bilateral p value
Gender (male)	32 (5)	40 (8)	**0.036**
Age (mean ± SD)	55.06 + 14.70	53.65 + 15.83	0.117
Dry mouth	598 (94)	466 (96)	0.209
Dry eyes	597 (94)	464 (96)	0.279
Altered ocular tests	534/591 (90)	420/450 (93)	0.091
Altered parotid scintigraphy	426/485 (88)	426/485 (88)	0.408
Positive salivary gland biopsy	265/310 (86)	220/247 (89)	0.253
Anemia (Hb <110 g/l)	88/631 (14)	114 (23)	**<0.001**
Leukopenia (<4000/mm^3)	109/632 (17)	104 (21)	0.078
Thrombocytopenia (<150,000/ mm^3)	33/631 (5)	52 (11)	**0.001**
Neutropenia (<1500/mm^3)	54/631 (9)	70 (14)	**0.002***
Lymphopenia (<1000/mm^3)	67/631 (11)	70 (14)	0.054
Antinuclear antibodies+	564/633 (89)	439/484 (91)	0.425
Rheumatoid factor +	283/609 (46)	282/473 (60)	**<0.001***
Anti-Ro/SS-A +	451/633 (71)	371/482 (77)	**0.033**
Anti-La/SS-B +	261/631 (41)	246/481 (51)	**0.001**
Monoclonal gammopathy	35/486 (7)	53/392 (13)	**0.002**
Cryoglobulins +	32/443 (7)	49/358 (14)	**0.003**
Low C3 levels (<0.82 g/l)	45/601 (7)	60/457 (13)	**0.003**
Low C4 levels (<0.11 g/l)	57/601 (9)	70/456 (15)	**0.004**
Baseline ESSDAI (mean ± SD)	4.20 + 5.06	8.12 + 7.98	**<0.001***

* Statistically significant ($p < 0.05$) in the multivariate analysis.

summarizes the association between the therapeutic approaches used and the outcomes. HCQ was associated with a lower risk of death (adjusted HR of 0.57, 95% 0.34–0.95) and development of lymphoma and/or death (adjusted HR of 0.55, 95% 0.34–0.88). Low doses of GCS were associated with a low risk of lymphoma (adjusted HR 0.42, CI 95% 0.19–0.92), while the use of high doses (>20 mg/day) or immunosuppressive agents was associated with a high risk of lymphoma, although statistical differences disappeared after adjustment (Fig. 2).

3.4. Adequacy

We classified 16 (7%) of the 255 patients treated with >20 mg of GCS as inadequately treated; in 9 patients, high doses of GCS were used to treat low ESSDAI activity (overwhelmingly articular), while in the remaining 7 patients, they were used to treat associated autoimmune features not included in the ESSDAI definitions (congenital heart block, uveitis, dacryoadenitis or autoimmune hepatitis). The use of immunosuppressive agents was classified as inadequate in 21/148 (14%) patients, once again overwhelmingly associated with articular involvement of low/moderate activity, while some patients were treated with IVIG/RTX for features not included in the ESSDAI (congenital heart block, orbital pseudotumor, and acquired C1 inhibitor deficiency).

4. Discussion

Primary SS is clearly Janus faced with respect to the outcome and prognosis. On the one hand, the majority of patients have a clinical presentation principally limited to the mucosal surfaces, together with fatigue and pain, without systemic involvement and/or laboratory prognostic markers [9]. On the other hand, a subset of patients present with high systemic activity and predictive immunological markers, and in these, a high risk of the development of lymphoma and/or death has recently been reported [10]. Measurement of systemic activity by the ESSDAI at the diagnosis of pSS is helpful in identifying this specific subset of patients who require closer follow-up and more-intensive therapeutic management [11]. In this study, we describe the main therapeutic options used for systemic SS in current clinical practice in a large series of patients, and we found that oral GCS were used in >40% of patients (more than half of whom received >20 mg/day), immunosuppressive agents in 13% and IVIG/RTX in <5% of patients. We found a close correlation between the use of these systemic therapies and systemic activity measured by the ESSDAI, confirming the usefulness of this score in daily practice, even when used retrospectively.
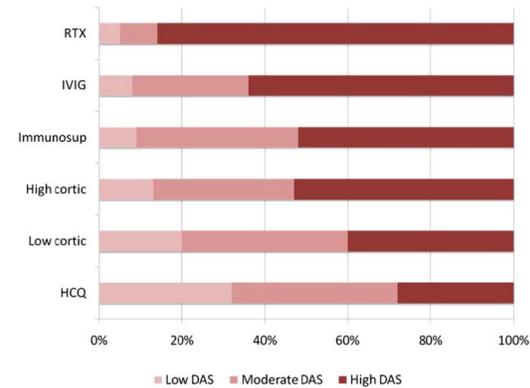

Fig. 1. Use of the different therapeutic approaches according to disease activity states (DAS) [8].

Table 3
Systemic therapies and poor outcomes in patients with primary SS.

Categories	n (%)	Death (N = 121)		Lymphoma (N = 28)[a]		Death and/or lymphoma (N = 138)[a]	
		Unadjusted HR	Adjusted HR[b]	Unadjusted HR	Adjusted HR[b]	Unadjusted HR	Adjusted HR[b]
a) Systemic drugs[c]							
Hydroxychloroquine (HCQ)	282 (25.2)	**0.58 [0.35–0.97]**	**0.57 [0.34–0.95]**	0.52 [0.18–1.50]	0.52 [0.18–1.53]	**0.60 [0.38–0.95]**	**0.55 [0.34–0.88]**
Corticoids <20 mg/day	475 (42.4)	1.33 [0.92–1.91]	1.09 [0.74–1.61]	0.82 [0.39–1.76]	**0.42 [0.19–0.92]**	1.08 [0.77–1.52]	0.79 [0.56–1.14]
Corticoids >20 mg/day	255 (22.8)	1.23 [0.82–1.85]	0.85 [0.55–1.31]	**2.40 [1.13–5.06]**	1.02 [0.46–2.23]	1.26 [0.86–1.83]	0.76 [0.51–1.14]
Immunosuppressive agents (ID)	148 (13.3)	1.06 [0.63–1.77]	0.83 [0.48–1.43]	**4.31 [2.02–9.21]**	1.96 [0.87–4.39]	1.29 [0.82–2.03]	0.93 [0.57–1.50]
Intravenous immunoglobulins (IVIG)	25 (2.2)	0.65 [0.16–2.64]	0.80 [0.20–3.27]	1.29 [0.18–9.54]	0.99 [0.13–7.37]	0.54 [0.13–2.20]	0.56 [0.14–2.27]
Rituximab (RTX)	35 (3.1)	0.83 [0.26–2.61]	0.44 [0.14–1.40]	NA	NA	**3.28 [1.77–6.08]**	1.47 [0.77–2.79]
b) Therapeutic schedules[c]							
No systemic therapies	635 (56.7)	REF	REF	REF	REF	REF	REF
HCQ and/or corticoids <20 mg/day	183 (16.3)	1.29 [0.80–2.06]	1.14 [0.70–1.86]	0.53 [0.12–2.38]	0.37 [0.08–1.70]	1.13 [0.72–1.78]	0.96 [0.61–1.53]
Corticoids >20 mg/day	132 (11.8)	1.49 [0.88–2.52]	0.96 [0.55–1.67]	0.75 [0.17–3.40]	0.41 [0.09–1.86]	1.16 [0.69–1.97]	0.67 [0.39–1.16]
ID, IVIG and/or RTX	170 (15.2)	1.22 [0.73–2.04]	0.87 [0.50–1.54]	**4.10 [1.84–9.16]**	1.46 [0.61–3.47]	1.44 [0.91–2.26]	0.87 [0.53–1.42]

The values are represented as the hazard ratios (HR) [95% confidence intervals].
REF: reference level.
In bold, statistically significant ($p < 0.05$) factors associated with death and/or lymphoma at univariate Cox proportional-hazards regression analysis.
NA: not applicable (rituximab is the main therapeutic option for the outcome analyzed).
[c] Separated univariate models were built for each treatment category considering non-treatment as the reference level.
　[a] Twenty-one patients with lymphoma were diagnosed before than fulfilling the 2002 classification criteria for primary SS and were excluded from the analysis. Two patients with unknown date of diagnosis were also excluded.
　[b] Adjusted for age at diagnosis, gender and levels of ESSDAI activity.

A quarter of our patients received HCQ during the follow-up, overwhelmingly for the treatment of articular involvement. HCQ has traditionally been associated with clinical benefits in fatigue and musculoskeletal pain, and uncontrolled studies have also found additional improvements in subjective and objective sicca features, reductions in parotid enlargement and oral infections and improvements in analytical and immunological parameters [6]. However, a recent double-blind, parallel-group, placebo-controlled trial reported that HCQ was no more effective than placebo for the main symptoms of pSS (dryness, fatigue and chronic pain) over 24 weeks of treatment [12]. In daily practice, we have used HCQ to treat other features and have found a close association with better survival, even after adjustment for gender, age and systemic activity, a similar result to that found in patients with SLE [13]. We recommend the use of HCQ for articular involvement associated with pSS (but not for associated fibromyalgia or chronic fatigue syndrome), always after ensuring optimal safety, especially with respect to adequate screening for retinal toxicity.

The use of GCS in clinical practice in pSS patients is not supported by reliable scientific evidence, since no study has specifically evaluated their use for extraglandular SS features [6]. In spite of this, our study found that they are used in >40% of patients. The use of systemic corticosteroids has been associated with a higher rate of adverse events, including increased appetite and weight gain and a two-fold higher frequency of diabetes mellitus [14]. Therefore, their use should be restricted to systemic features with at least moderate/severe ESSDAI activity. However, we found an inadequate use of GCS in nearly 10% of our cases, especially in patients with arthralgia: this should be corrected.

The use of immunosuppressive agents in pSS is based on the same level of evidence as that of GCS. Several studies have analyzed the use of CyA, AZA, methotrexate, leflunomide and mycophenolic acid in small (<25) series of patients [5]. These studies have provided three key messages: limited benefits for sicca features, the lack of a specific analysis of extraglandular features and an unacceptable rate of adverse events. We found that, in clinical practice, immunosuppressive agents were used for refractory moderate systemic involvement (mainly arthritis, extensive cutaneous purpura, and non-severe peripheral

neuropathy) and in patients with internal organ involvement (interstitial lung disease, glomerulonephritis, or severe neurologic features). The most-frequently used immunosuppressive agents are azathioprine/mycophenolate in interstitial lung disease, methotrexate in joint involvement and cyclophosphamide for glomerulonephritis, vasculitis, multiple neuritis and central nervous system involvement. Our results showed an inadequate use of immunosuppressive agents in 14% of cases, mainly in patients with moderate arthritis. In these patients, we recommend an exhaustive diagnostic approach, discarding synovitis due to osteoarthritis, non-autoimmune causes of arthritis (mainly infectious and metabolic) and osteomalacia caused by renal tubular acidosis.

The emergence of biological therapies has increased the therapeutic armamentarium available for treating SS, but their use is limited by the lack of licensing [5]. Random controlled trials (RCT) have demonstrated the lack of efficacy of anti-tumor necrosis factor agents and promising results for B-cell depleting agents [6]. Four recent studies evaluated the use of RTX in pSS patients. Gottenberg et al. [15] reported on the use of RTX in 78 patients with refractory systemic SS and found an overall efficacy of 60%, while Carubbi et al. [16] found a faster and more-pronounced reduction of systemic activity in patients treated with RTX than in those treated with conventional therapy. In contrast, the efficacy of RTX for non-systemic features (the triad of dryness, fatigue and pain) is not so clear. St Clair et al. [17] found only modest improvements in a small open-label trial of 12 patients treated with RTX, while recent data from a French RCT including 120 patients found no significant changes [18].

The great influence of personal and environmental factors on the intensity of these symptoms, which are measured subjectively using visual analogue scales, may account, in part, for the lack of significant differences. We used RTX for systemic involvements refractory to standard treatment (lack of response or intolerance to corticosteroids and immunosuppressive agents) [19], including severe, life-threatening involvement involving vital organs such as the kidneys, the lungs and the gastrointestinal tract, progressive ataxic neuronopathy, severe cytopenia and lymphoma [20]. Although the amount and quality of evidence on the off-label use of RTX in SS-related extraglandular

Fig. 2. Non-adjusted and adjusted Kaplan–Meier curves (adjusted for age at diagnosis, gender and levels of baseline ESSDAI activity).a) Ever use of hydroxychloroquine during the follow-up as predictor of death (left: non-adjusted, right: adjusted).b) Ever use of hydroxychloroquine during the follow-up as predictor of poor outcome (lymphoma and/or death). Left: non-adjusted, right: adjusted.c) Ever use of corticoids <20 mg/day during the follow-up as predictor of lymphoma (left: non-adjusted, right: adjusted).d) Ever use of immunosuppressive agents during the follow-up as predictor of lymphoma (left: non-adjusted, right: adjusted).

a) Ever use of hydroxychloroquine during the follow-up as predictor of death (Left: non-adjusted, Right: adjusted)

b) Ever use of hydroxychloroquine during the follow-up as predictor of poor outcome (lymphoma and/or death). Left: non-adjusted, Right: adjusted

c) Ever use of corticoids < 20 mg/d during the follow-up as predictor of lymphoma (Left: non-adjusted, Right: adjusted).
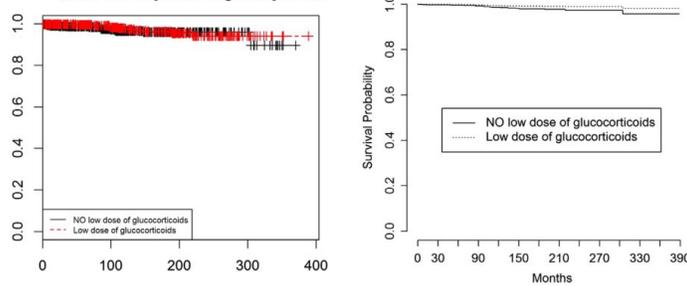
d) Ever use of immunosuppressive agents during the follow-up as predictor of lymphoma (left: non-adjusted, right: adjusted).

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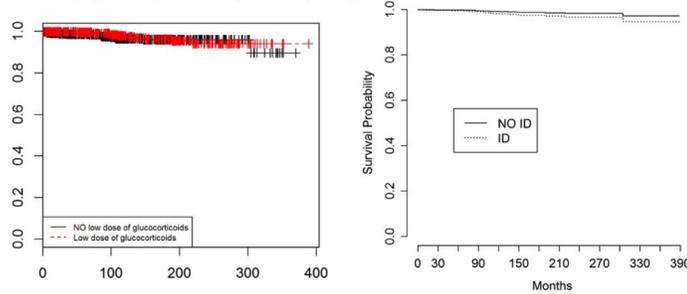
features are higher than that reported for the use of the standard options (corticosteroids and immunosuppressive agents), a reasonable assessment of the risk of serious adverse events versus the benefits of treatment should be always made on an individual basis. We consider that the off-label use of these biological agents to treat only dryness, fatigue and/or pain (even when severe) is not warranted.

In summary, the management of systemic Sjögren should be organ-specific, using low doses of corticoids in patients with moderate systemic activity (including HCQ for articular involvement), and limiting the use of high doses of corticoids to potentially severe scenarios, while HCQ may be used in patients with articular involvement. Immunosuppressive agents, IVIG and RTX should be limited to systemic involvements, either in patients refractory to corticosteroids or in life-threatening situations and, due to their off-label use, always with a reasonable assessment of the risk of serious adverse events versus the benefits of treatment. The use of systemic therapies for dryness, chronic pain or fatigue is not warranted.

Acknowledgments

Appendix 1

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Treating the Underlying Pathophysiology of Primary Sjögren Syndrome: Recent Advances and Future Prospects

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Abstract Sjögren Syndrome (SS) is a systemic autoimmune disease with a wide clinical spectrum that extends from sicca symptoms of the mucosal surfaces to extraglandular systemic manifestations. Understanding of the pathophysiology of primary SS has advanced over recent years, and this, in turn, has presented new targeted treatment options. We provide a brief, up-to-date description of the pathophysiology of SS and the main etiopathogenic pathways implicated in the disease process and review clinical evidence in support of new treatment options targeting these pathways, highlighting successes and failures, and concluding with a summary of gaps in knowledge and where future research should be focused. Direct and indirect B-cell targeted therapies are currently the most promising biological agents in primary SS, especially for systemic involvement, but other pathways (T-cell co-stimulation, cytokine-based therapies, intracellular pathways and gene therapies) are under development. The next 10 years may witness a disruptive therapeutic scenario in primary SS.

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Significant etiopathogenic advances beyond B cells are opening up new therapeutic approaches in primary Sjögren Syndrome (SS).

Currently, B-cell targeted therapies are the biological agents with the most solid scientific basis for use in primary SS, although still on a case-by-case, off-label regimen.

Therapies targeting T-cell co-stimulation, cytokines, intracellular pathways or genes may be game-changers.

1 Introduction

Sjögren Syndrome (SS), a systemic autoimmune disease overwhelmingly diagnosed in women aged more than forty years, has a frequency of around 0.1% and an incidence rate of around 1 new case per 10,000 persons [1]. Clinically, patients may present with sicca symptoms (caused by exocrine gland involvement), general features (dominated by fatigue and chronic pain) and systemic extra-glandular involvement (including lymphoma) (Table 1). Glandular involvement is confirmed by specific oral (salivary flow measurement, parotid scintigraphy) and ocular (fluorescein staining, Schirmer test) diagnostic tests [2]. The histological hallmark of SS (focal lymphocytic infiltration) is demonstrated by minor labial salivary gland biopsy [3]. Patients present a broad spectrum of analytical abnormalities (mainly cytopenias) and a plethora of circulating autoantibodies, of

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Table 1 List of the main glandular and extra-glandular manifestations of primary Sjögren Syndrome

Organ	Manifestation
Oral symptoms	Hyposalivation, soreness, dysphagia, difficulties in speaking or eating, dental caries, oral candidiasis
Ocular symptoms	Foreign-body sensation, conjunctival inflammation, eye fatigue, decreased visual acuity, blepharitis, bacterial keratitis
General symptoms	Fatigue, chronic pain, fever
Skin	Cutaneous dryness, purpura, cutaneous ulcers, annular erythema
Articular	Arthralgias, arthritis
Lungs	Chronic cough, obstructive lung disease, bronchiectasis, interstitial lung diseases
Cardiovascular	Raynaud phenomenon, pulmonary arterial hypertension, dysautonomia
Pancreas	Recurrent acute pancreatitis
Nephro-urological	Renal tubular acidosis, glomerulonephritis, interstitial cystitis
Peripheral nerves	Polyneuropathy, multineuritis, ataxic neuronopathy
Central nervous system	MS-like disease, cranial nerve involvement, neuromyelitis optica, recurrent meningitis
Obstetrics	Autoimmune congenital heart block, neonatal lupus
ENT	Recurrent parotid enlargement, neurosensorial hearing loss

ENT ear nose throat, *MS* multiple sclerosis

which antinuclear antibodies are the most frequently detected, anti-Ro/SS-A and anti-La/SS-B the most specific, and cryoglobulins the main prognostic marker; other frequently-detected immunological markers are rheumatoid factor (40–60%) and hypocomplementemia (10–20%).

The treatment of primary SS (pSS) has two principal objectives: symptomatic management of sicca manifestations and broad-spectrum immunosuppression (including glucocorticoids and immunosuppressive agents) to treat systemic involvement [4]. Unfortunately, studies analyzing the effects of immunosuppressive agents in patients with primary SS, which are overwhelmingly uncontrolled, are designed to evaluate sicca rather than systemic outcomes [4]. The emergence of biological therapies has increased the therapeutic armamentarium available to treat pSS [5], but their use is still limited by a lack of licensing. This review summarizes the current pharmacotherapy options targeting the main mechanisms involved in the etiopathogenesis of pSS and future directions for the development of new therapies.

2 Etiopathogenesis of Primary SS: A Brief Update

A broad etiopathogenic hypothesis of the genesis of pSS suggests that multiple environmental factors affecting an individual with a specific genetic susceptibility may be at the origin of the disease. A recent study has estimated a heritability of 54% [6]; however, although the prevalence of pSS in relatives of patients with the disease was 12-fold higher than that of the general population, the authors estimated that at least 80% of cases of pSS may be sporadic. Genetic susceptibility to pSS is complex, and large-scale genome-wide association studies (GWAS) published since 2013 have reported a strong association with the HLA Class I and II loci (A, C, DRB6, DPB1, and DQA1) [1].

In 1994, Harry Moutsopoulos proposed what remains the most widely accepted etiopathogenic model, based on autoimmune epithelitis as the key underlying pathogenic scenario [7], in which both pSS-related exocrinopathy and extra-glandular involvement are characterized by the infiltration of lymphocytes around or invading the exocrine epithelium, the key tissular target of the autoimmune response. In this model, the epithelium is not just an innocent bystander "suffering" the infiltration of the autoimmune cells, but plays an active, central role in regulating the autoimmune mechanisms, acting as an antigen-presenting tissue [1]. The innate immune system plays an important role in early stages of the etiopathogenic process, notably through activation of the type I interferon (IFN) system by external factors [8]. Viruses are suspected to be the main triggers through their interaction with host pattern recognition receptors (PRRs) such as toll-like receptors (TLR). The response of T and B cells to the autoantigens abnormally expressed by the epithelium of the exocrine glands (Ro and La antigens) initiates cytokine and chemokine synthesis, leading to chronic inflammatory damage to the exocrine glands and, finally, the loss of their physiological functions [9–11].

Autoimmune epithelitis is one etiopathogenic hypothesis in pSS. Other studies have suggested a key role for neuroendocrine mechanisms that could explain why some pSS patients present with severe sicca syndrome with no (or limited) inflammatory histopathological features [12–15], involving hypoactivity of the hypothalamic-pituitary-adrenal (HPA) axis due to a pituitary defect or adrenal

gland dysfunction [12], or to lack of estrogens (which has been associated with autoimmune exocrinopathy in experimental models) [13]. Reduced secretory function has been related to the destruction of neural innervation of the glands, the absence of acetylcholine receptors of the glandular cells, the role of inflammatory cytokines that could reduce the release of neurotransmitters, and to the decreased response of the residual glandular cells to available neurotransmitters [14]. Other etiopathogenic pathways recently associated with sialadenitis include autophagy, the role of aromatases and, especially, epigenetic mechanisms involving the modification of histones, non-coding RNAs and DNA methylation [1, 16].

Recent studies have focused on the effect of environmental factors in the pathogenesis of pSS. One example is the potential role of the human microbiota, which consists of the 10–100 trillion symbiotic microbial cells harbored by each person [17]; in pSS, the first studies have centered on the role of the ocular and oral microbiota [18, 19]. Another fresh line of etiopathogenic investigation is the potential effect of exposure to environmental toxins, including occupational toxins (solvents, aerosolized dust) [20] and airborne pollution (fine particulates) [21].

3 Etiopathogenic-Based Therapeutic Approaches

The most recent etiopathogenic advances in patients with pSS involve new, highly-selective biological therapies without the adverse effects often associated with standard, less-selective therapeutic options (corticosteroids, immunosuppressive drugs). The emergence of biological agents targeting molecules and receptors involved in the etiopathogenesis of pSS has presented a new era in the therapeutic management of the disease.

3.1 B-Cell Targeting

3.1.1 Direct B-Cell Blocking

B-cell targeted agents remain the most promising biological therapies in pSS (Table 2). Monoclonal antibodies to

Table 2 Use of B-cell targeted therapies in patients with primary Sjögren Syndrome: main studies

Author (y)	B-cell therapy	Study design	Number of patients with primary SS
Dass (2008) [79]	Rituximab	RCT	17
Meijer et al. (2010) [80]	Rituximab	RCT	30
Meiners et al. (2014) [46]		Substudy	
Meiners et al. (2015) [81]		Extension study	
Devauchelle-Pensec et al. (2014) [29]	Rituximab	RCT	122
Bowman et al. (2015) [82]	Rituximab	RCT	133
Pijpe et al. (2005) [83]	Rituximab	Open-label	15
Devauchelle-Pensec et al. (2007) [84]	Rituximab	Open-label	16
Meiners et al. (2012) [85]	Rituximab	Open-label	28
Mekinian et al. (2012) [86]	Rituximab	Open-label	17
Carubbi et al. (2013) [87]	Rituximab	Open-label	41
St. Clair et al. (2013) [88]	Rituximab	Open-label	12
Gottenberg et al. (2005) [89]	Rituximab	Retrospective	6
Seror et al. (2007) [90]	Rituximab	Retrospective	16
Vasil'ev et al. (2009) [91]	Rituximab	Retrospective	10
Ramos-Casals et al. (2010) [92]	Rituximab	Retrospective	24
Tony et al. (2011) [93]	Rituximab	Retrospective	4
Gottenberg et al. (2013) [94]	Rituximab	Retrospective	74
Zhou et al. (2012) [95]	Rituximab	Retrospective	4
Mekinian et al. (2012) [96]	Rituximab	Retrospective	11
Pollard et al. (2011) [97]	Rituximab	Retrospective	19
Voulgarelis et al. (2012) [98]	Rituximab	Retrospective	17
Jiang et al. (2015) [31]	Rituximab	Retrospective	6
Steinfeld et al. (2006) [34]	Epratuzumab	Open-label	16
Mariette et al. (2015) [40]	Belimumab	Open-label	30
De Vita et al. (2015) [42]		Extension study	

RCT randomized controlled trial, *SS* Sjogren's Syndrome, *y* year

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B-cell receptors can induce or block B cells from entering the cell cycle and induce or block apoptosis; these different outcomes may depend on the B-cell stage and the receptor blocked [22]. Rituximab was, in 1997, the first anti-B-cell receptor monoclonal antibody approved by the U.S. Food and Drug Administration (FDA) to treat B-cell lymphomas. Rituximab blocks the CD20 lymphocytic receptor, leading to complete B-cell depletion in blood; after a first cycle of rituximab, higher proportions of naïve B cells than memory B cells return. Rituximab also initially reduces specific subsets of T-cells, particularly CD4+ and CD8+ T-cells, due to changes in cytokines leading to redistribution or decreased trafficking of T-cells, reduced numbers of antigen-presenting cells and co-stimulatory cell surface markers available to promote T-cell differentiation, and/or indirect reductions in the frequency of regulatory T-cells [23]. Rituximab is associated with changes in mean counts of other cell types, such as neutrophils [24].

In pSS, the consequences of rituximab therapy include complete B-cell depletion in serum, partial depletion of B cells in the salivary glands [25] and an effect on the T-cell arm by reducing the absolute numbers of T follicular helper (Tfh) cells [1]. In addition, rituximab modulates interleukin (IL)-17 expression in the salivary glands of patients with pSS and, after rituximab therapy, salivary gland expression of IL-17 is significantly reduced and accompanied by pronounced apoptotic depletion of mast cells [26]. IL-17-producing pathogenic T lymphocytes co-express CD20 and are also depleted by rituximab in pSS [27].

Since 2005, more than 20 uncontrolled studies have evaluated rituximab in patients with pSS, including retrospective studies (Table 3), open-label prospective studies (Table 4) and randomized controlled trials (RCTs) (Table 5), evaluating either systemic involvement or B-cell lymphoma [28]. Until now, rituximab has been used in more than 600 patients included in controlled or uncontrolled studies, with a wide range of outcomes evaluated, including sicca features, fatigue and, especially, systemic features and lymphoma. The amount and quality of evidence on the off-label use of rituximab to treat pSS-related extra-glandular features is higher than that reported for standard options (corticosteroids and immunosuppressive agents). In spite of these promising results, a recent multicenter RCT [29] evaluated 122 consecutive patients assigned to rituximab infusions (1 g) or placebo at weeks 0 and 2 and found no significant differences in the primary outcome (a composite score that evaluated dryness, pain, fatigue and global health at 24 weeks), objective diagnostic tests (Schirmer test, salivary gland biopsy) or improvement in the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) score, although significant differences were found for some secondary endpoints [sicca and fatigue visual analogue scale (VAS) and salivary flow rate]. In

addition, a recent systematic review and meta-analysis that evaluated the effectiveness and safety of rituximab found, with a low quality of evidence, that studies have reported no efficacy at 24 weeks with respect to oral dryness VAS, fatigue reduction, improved quality of life or disease activity [30]. The off-label use of rituximab to treat only these symptoms (dryness, pain, fatigue) is not currently warranted [4].

In contrast, rituximab is the most widely used off-label biological agent in patients with severe systemic involvement refractory to standard treatment and is increasingly used in patients with severe cytopenias refractory to conventional immunosuppressive agents [31] and in patients with associated B-cell lymphoma [1]. Therefore, current scientific evidence suggests that the off-label use of rituximab may be considered in patients with involvements refractory to standard treatment (lack of response or intolerance to corticosteroids and immunosuppressive agents) [32]. The great majority of studies of the treatment of systemic features are uncontrolled, while RCTs included the effect of the drug on sicca and non-specific general features as primary outcomes; this might partially explain the negative results reported by RCTs.

Other anti-CD20 monoclonal antibodies include ocrelizumab (humanized B-cell-depleting agent), ofatumumab (HuMax-CD20, a fully human B-cell-depleting agent) and obinutuzumab (a glycol-engineered Fc fragment that enhances binding affinity to the FcγRIII receptor on immune effector cells) (Fig. 1). The type II antibody binding characteristics of obinutuzumab to CD20 lead to more efficient induction of direct non-apoptotic cell death. Obinutuzumab has an acceptable safety profile in patients with B-cell lymphoma, with infusion-related reactions during the first infusion being the most common adverse event [33].

Epratuzumab targets CD22 B-cell antigen receptor signaling, leading to partial B-cell depletion. A beneficial effect on fatigue was observed in a small prospective study, but the effect on extra-glandular manifestations was not evaluated [34] and no further studies were made.

3.1.2 Indirect B-Cell Blocking

The B-cell activating factor axis comprises two ligands [B-lymphocyte stimulator

(BLyS)/BAFF and APRIL] and three receptors (BCMA, TACI, BR3) [35]. BLyS is a critical cytokine involved in the survival of circulating B cells [36]. Its binding to the corresponding receptors inhibits intracellular apoptotic pathways and provides survival signals for B cells [37]. Quartuccio et al. [38] found higher sBLyS levels in patients with pSS, which were closely associated with the main immunological markers, systemic disease activity and

Table 3 Retrospective studies using rituximab in patients with Sjögren Syndrome evaluating systemic outcomes and use of prednisone

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Lymphoma (clinical response)	Systemic involvement (response)	Prednisone use	Serological response
Gottenberg et al. (2005) [89]	6 (6)	57.5	Retrospective (8 mo) (32 w)	Rituximab 375 mg/m² w 0, 1, 2, 3 (n = 5) w 0,1 (n = 1)	MALT (1/2)	Systemic (n = 4) Vasculitis (2/2) Parotid + arthritis (2/2)	Reduction in 4/5	RF reduction 4/4 Cryo negative 2/2
Seror et al. (2007) [90]	16 (16)	58	Retrospective 4.5 mo (16 w)	Rituximab 375 mg/m² w 0, 1, 2, 3 (n = 14) Other regimens (n = 2)	Dryness improv 2/11 (18%)	Systemic features 9/11 (82%) Cryo 4/5 Thrombocytopenia 0/1 Pulmonary + arthritis 2/2 Arthritis 2/2 Renal 1/1 Parotid enlargement 3/3	Reduced median daily dose of corticosteroids (0.003)	ESR (p = 0.009), CRP (p = 0.02), gamma (p = 0.003), beta2 (p = 0.003), cryo negative 4/4, reduced RF (p = 0.004)
Vasil'ev et al. (2009) [91]	13 (ND) 3 associat	ND	Retrospective (ND)	Rituximab ND 500 mg MP premedic (n = 13)	Lymphoma CR 7, PR 2	Systemic (3/4)	ND	ND
Ramos-Casals et al. (2010) [92]	15 (15)	58	Retrospective Therapeutic response to RTX was evaluated at 12 mo (48 w)	Rituximab 375 mg/m² w 0, 1, 2, 3 (n = 11) Rituximab 1 g w 0, 2 (n = 4)	Lymphoma (n = 8): CR in 6, PR in 2	Systemic (n = 11) CNS 1/3 PN 5/5 Cytopenia 3/3 GN 1/2 Arthritis 0/1 Muscular 1/1 LPE 1/1	ND	ND
Tony et al. (2011) [93]	4 (ND)	ND	Retrospective (ND)	ND	ND	ND PR (n = 2), CR (n = 2)	ND	ND

Table 3 continued

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Lymphoma (clinical response)	Systemic involvement (response)	Prednisone use	Serological response
Gottenberg et al. (2013) [94]	78 (67)	59,8	Retrospective (ND)	Rituximab 375 mg/m² w 0, 1, 2, 3 (n = 11) Rituximab 1 g w 0, 2 (n = 67)	ND	Systemic (44/74) Articular 17/27 (63%) CNS 2/6 (33%) PN 6/12 (50%) Lung = 7/9 (78%) Vasculitis = 5/8 (63%) Renal = 5/6 (83%) Myositis = 0/3 (0%) Cytopenia = 2/2 (100%) Pancreatitis = 1/1 (100%) Glandular enlarge = 2/3 (67%) Sclera vasculitis 0/1 (0%) ESSDAI score (p < 0.0001)	Reduced median daily dose of corticosteroids mg/day (p = 0.1)	ND
Jiang et al. (2015) [31]	6 (6)	37	Retrospective Therapeutic response to RTX was evaluated at 12 mo (48 w)	Rituximab 200 mg x1 (n = 1) Rituximab 100 mg x1, w 0, 1 (n = 1) Rituximab 200 mg x1, w 0, 1 (n = 1) Rituximab 500 mg x1, w 0, 1 (n = 3)	–	AITP	ND	CR (n = 5) PR (n = 1)

d day, *mo* months, *n* number, *w* weeks, *y* years, *NS* no significant differences, *ND* not detailed, *MP* methylprednisolone, *improv* improvement, *premedic* premedication, *associat* associated, *ESR* erythrosedimentation rate, *CRP* C-reactive protein, *beta2* microglobulin, *cryo* cryoglobulins, *RF* rheumatoid factor, *GN* glomerulonephritis, *CNS* central nervous system, *PN* polyneuropathy, *AITP* autoimmune thrombocytopenia, *CR* complete response, *PR* partial response, *MALT* mucosa-associated lymphoid tissue, *ESSDAI* EULAR Sjögren's Syndrome Disease Activity Index, *LPE* losing-protein enteropathy

Table 4 Open-label studies evaluating B-cell targeted therapies (rituximab, epratuzumab, belimumab) in patients with primary Sjögren Syndrome

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Outcomes evaluated	Significant differences
Pijpe et al. (2005) [83]	15 (14)	50	Prospective 12 w	Rituximab 375 mg/m² Weeks 0, 1, 2, 3 (n = 15)	Unstimulated flow rate Sialochemical analysis Schirmer, RB, BUT Subjective VAS MFI, SF-36 questionnaire	Early pSS group RB score ($p < 0.05$), BUT ($p < 0.05$), MFI ($p < 0.05$ 4/5 scales), SF-36 ($p < 0.05$ 5/9 scales) MALT/pSS group RB score ($p < 0.05$)
Devauchelle-Pensec et al. (2007) [84]	16 (14)	55	Prospective 36 w	Rituximab 375 mg/m² Weeks 0, 1 (n = 16)	VAS global, pain, dryness, fatigue Tender joints Unstimulated flow rate Schirmer, van Bijsterveld ESR, CRP, abs, IgG Salivary gland biopsy SF-36	Global VAS ($p = 0.03$), VAS pain ($p = 0.006$), fatigue VAS ($p = 0.006$), dryness VAS ($p = 0.006$) Tender point ($p = 0.027$) and joint ($p = 0.017$) count – – RF reduction ($p = 0.04$) – Mental and physical components ($p = 0.03$) Resolution pulmonary involv ($n = 1$)
Meiners et al. (2012) [85]	28 (27)	43	Prospective 60 w	Rituximab 1 g/15 days Weeks 0, 2 (n = 28)	ESSDAI ESSPRI Patient's GDA Physician's GDA IgM-RF SWS	ESSDAI (w16 [$p = 0.000$], w24 [$p = 0.000$], 36w [$p = 0.000$]) ESSPRI (w16 [$p = 0.000$], w24 [$p = 0.001$], 36w [$p = 0.007$], 60w [$p = 0.043$]) Patient's GDA (w16 [$p = 0.000$], w24 [$p = 0.000$], 36w [$p = 0.000$], 48w [$p = 0.000$], 60w [$p = 0.010$]) Physician's GDA (w16 [$p = 0.000$], w24 [$p = 0.000$], 36w [$p = 0.000$], 48w [$p = 0.000$], 60w [$p = 0.000$]) IgM-RF (w16 [$p = 0.000$], w24 [$p = 0.000$], 36w [$p = 0.000$])
Mekinian et al. (2012) [86]	17 (14)	60	Prospective 33 mo (132 w)	Rituximab 1 g/15 days Weeks 0, 2 Group 1: patients with cryoglobulinemia or vasculitis (n = 10) Group 2: patients with neither cryoglobulinemia nor vasculitis (n = 7)	Efficacy PNS involvement PR or CR and/or electrophysiological improvement at 3, 6 and 9 mo Modified Rankin scale ESSDAI	Neurological improvement in 11 patients (65%) at 3 mo Sensorimotor neuropathy ($p < 0.05$) Multineuritis ($p = 0.07$) Rankin scale ($p = 0.02$) ESSDAI ($p < 0.05$) Rituximab was effective in neurological involvement in 9/10 patients with vasculitis or cryoglobulinemia (90%) (group 1) at 3 mo and in 2/7 cases (29%) without Cryoglobulinemia and vasculitis ($p = 0.03$)

Table 4 continued

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Outcomes evaluated	Significant differences
Carubbi et al. (2013) [87]	41 (40)	40 (RTX) 43 (DMARDs)	Prospective 120 w	Rituximab 1 g/15 days Weeks 0, 2 Every 24 w ($n = 19$) DMARDs (HCQ, MTX, Cyc) ($n = 22$)	Efficacy (primary endpoint) Global disease activity VAS Pain VAS Fatigue VAS Dryness VAS Physician GA VAS USF (mL/minute) Schirmer I test (mm/ ESSDAI MSGs Ig G, abs, RF	Clinical efficacy at 120 w Global disease activity VAS (w 48, 72, 96, 120 $p < 0.001$) Pain VAS (w 48, 72, 96, 120 $p < 0.001$) Fatigue VAS (w 48, 72, 96, 120 $p < 0.001$) Dryness VAS (w 48 $p < 0.01$, 72, 96, 120 $p < 0.001$) Physician GA VAS (w 48 $p < 0.01$, 72, 96, 120 $p < 0.001$) USF (mL/minute) (w 48 $p < 0.01$, 72, 96, 120 $p < 0.001$) Schirmer-I test (mm/5 min) (w 72, 96, 120 $p < 0.001$) ESSDAI (w 48, 72, 96, 120 $p < 0.001$) MSGs: percentage of CXCR4- positive cells and CXCR5-positive cells inside the glandular infiltrate $p < 0.001$. Reduction germinal centers (GCs) in rituximab group at week 120 NS
St. Clair et al. (2013) [88]	12 (12)	51	Prospective 52 w	Rituximab 1 g/15 days Weeks 0, 2 ($n = 12$)	Safety (primary endpoint) VAS physician/patient VAS sicca/fatigue Ocular tests Salivary flow rates SF-36 questionnaire Autoantibodies	Clinical efficacy at 26 w VAS physician ($p = 0.012$) and patient ($p = 0.009$) VAS tongue dryness ($p = 0.007$), level of thirst ($p = 0.005$), oral discomfort ($p = 0.02$), fatigue ($p = 0.042$) – – Vitality ($p = 0.006$) –
Steinfeld et al. (2006) [34]	16 (14)	49	Prospective 18 w	Epratuzumab (360 mg/m²) at 0, 2, 4, and 6 w ($n = 15$)	Schirmer-I test Unstimulated whole salivary flow Fatigue and pain VAS Patient assessment Physician assessment ESR IgG ≥20% clinical improvement in at least two of the aforementioned parameters with ≥20% reduction in ESR and/or IgG considered as a single combined criterion	Schirmer-I test: improved in 73, 69, 64, and 64% at 6, 10, 18, and 32 w, respectively Unstimulated whole salivary flow: improved in 20, 46, 36, and 64% at 6, 10, 18, and 32 w, respectively Fatigue VAS ($p < 0.05$) Patient assessment ($p < 0.05$) Physician assessment ($p < 0.05$) ESR: improved in 33% at 18 w IgG: improvement was seen in 13, 7, and 20% of the patients at 10, 18, and 32 weeks, respectively 53% achieved a clinical response (≥20% improvement) at 6 weeks, with 53, 47, and 67% responding at 10, 18, and 32 weeks, respectively

Table 4 continued

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Outcomes evaluated	Significant differences
Mariette et al. (2015) [40]	30 (30)	49.5	Prospective 24 w	Belimumab, 10 mg/kg, Week 0, 2, 4 and then every 4 w to W 24 (n = 30)	Efficacy and safety (primary endpoint) Improvement in two of five items: ≥30% reduction in dryness score on a VAS; ≥30% reduction in fatigue score on a VAS; ≥30% reduction in musculoskeletal pain score on a VAS; ≥30% reduction in systemic activity score on a VAS assessed by the physician; ≥25% reduction in serum levels of any of the following B-cell activation biomarkers (free light chains of immunoglobulin, βeta2, monoclonal component, cryo, IgG); ≥25% increase in C4 level; Secondary endpoints: Schirmer's test; USF; ESSDAI; ESSPRI; SF36	Primary endpoint in 18 (60%); Dryness VAS ($p = 0.0021$); Fatigue VAS ($p = 0.0606$); Pain VAS ($p = 0.89$); Secondary endpoints: Schirmer's test (NS); USF (NS); ESSDAI ($p = 0.0015$); ESSPRI ($p = 0.0174$); SF36 (NS)
De Vita et al. (2015) [42]	19 (19)	40	Prospective 52 w (extension study, Mariette 2015)	Belimumab, 10 mg/kg, Week 0, 2, 4 and then every 4 weeks to W 52 (n = 19)	Efficacy and safety (primary endpoint) Improvement in two of five items: ≥30% reduction in dryness score on a VAS; ≥30% reduction in fatigue score on a VAS; ≥30% reduction in musculoskeletal pain score on a VAS; ≥30% reduction in systemic activity score on a VAS assessed by the physician; ≥25% reduction in serum levels of any of the following B-cell activation biomarkers (free light chains of Igs, βeta2, monoclonal component, cryo, IgG); ≥25% increase in C4 level; Secondary endpoints: Schirmer's test; USF; ESSDAI; ESSPRI; SF36	Primary endpoint in 13/15 (86.7%); Dryness VAS (NS); Fatigue VAS (NS); Pain VAS (NS); Secondary endpoints: Schirmer's test (NS); USF (NS); ESSDAI 28 w vs. 52 w ($p = <0.0001$); ESSPRI ($p = 0.01$); SF36 (NS)

pSS primary Sjögren Syndrome, *mo* months, *n* number, *w* weeks, *y* years, *NS* no significant differences, *ND* not detailed, *mg* miligrams, *Kg* kilogram, m^2 square meter, *invol* involvement, *MALT* mucosa-associated lymphoid tissue, *VAS* visual analogue scale, *RB* rose bengal staining, *BUT* break-up time, *USF* unstimulated salivary flow, *ESR* erythro-sedimentation rate, *CRP* C-reactive protein, *beta2* beta2 microglobulin, *cryo* cryoglobulins, *RF* rheumatoid factor, *Igs* serum immunoglobulins, *C* complement, *abs* autoantibodies, *C* complement, *GA* global assessment, *MFI* Multidimensional Fatigue Inventory, *SF-36* Medical Outcomes Short Form, *ESSPRI* European League Against Rheumatism (EULAR) Sjögren's Syndrome Patient Reported Index, *ESSDAI* EULAR Sjögren's Syndrome Disease Activity Index, *GDA* global disease activity, *MSGs* minor salivary glands, *DMARDs* disease modifying antirheumatic drugs, *HCQ* hydroxychloroquine, *MTX* methotrexate, *Cyc* cyclosporine, *RTX* rituximab, *PNS* peripheral nervous system, *CR* complete response, *PR* partial response

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Table 5 RCT evaluating rituximab in patients with primary Sjögren Syndrome

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Primary outcome (results)	Secondary outcomes (significant differences)
Dass et al. (2008) [79]	17 (ND)	52	RCT-d 6 mo (24 w)	Rituximab 1 g/15 days ($n = 8$) Placebo ($n = 9$) Week 0 and 2	Fatigue VAS improvement >20% at 6 mo (87% vs. 56%, $p = 0.36$)	VAS: fatigue (<0.001), general health ($p = 0.021$) SF-36: social functioning ($p = 0.01$) PROFAD VAS, FCIT-F Schirmer test Unstimulated flow rate ESR, CRP, abs, IgG, RF reduction ($p = 0.05$)
Meijer et al. (2010) [80]	30 (29)	43	RCT-d 48 w	Rituximab 1 g/15 days ($n = 20$) Placebo ($n = 10$) Weeks 0 and 2	Improvement of stimulated whole salivary flow rate at 5, 12, 24 and 48 weeks ($p = 0.038$ only at 12 w)	Salivary flow rates: SWS at 12 w ($p < 0.05$) Ocular tests: LISS ($p < 0.05$) Igs, decreased RF ($p < 0.05$) VAS: dry mouth night ($p < 0.05$), dry eyes ($p < 0.05$) SF-36: vitality ($p = 0.013$) MFI: reduced activity ($p = 0.023$) Extra-glandular features: decreased number (0.029), reduced vasculitis ($p = 0.03$)
Devauchelle-Pensec et al. (2014) [29]	122 (97)	53	RCT-d 24 w	Rituximab 1 g/15 days ($n = 63$) 2 infusions of rituximab ($n = 58$) 1 infusion of rituximab ($n = 5$) Placebo ($n = 57$) 2 infusions of placebo ($n = 55$) 1 infusion of placebo ($n = 1$) 1 infusion of rituximab and 1 of placebo ($n = 1$)	NS between groups in the primary endpoint was found (difference, 1.0% [95% CI, −16.7 to 18.7%])	At least two of the four VAS scores were higher in the rituximab group at week 6 (22.4 vs. 9.1%; $p = 0.036$) An improvement of at least 30 mm in VAS fatigue score was more common with rituximab at weeks 6 ($p < 0.001$) and 16 ($p = 0.012$) IgG, mg/dL ($p = 0.003$), IgA, mg/L ($p = 0.026$), IgM, mg/L ($p = 0.004$)
Meiners et al. (2014) [46]	30 (29)	43	RCT-d 24 w (substudy Meijer et al. 2010)	Rituximab 1 g/15 days ($n = 20$) Placebo ($n = 10$) Weeks 0 and 2	ESSDAI was lower in the rituximab group at weeks 12 and 24	–
Bowman et al. (2015) [82]	133 (70)	53	RCT-d 48 w	Rituximab 1 g/15 days ($n = 67$) Placebo ($n = 66$) Weeks 0, 2, 24, 26	Response rates were 39.8% (OR 1.13, 95% CI 0.50–2.55) There were NS in any outcome measure, except SWS	–

Table 5 continued

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Primary outcome (results)	Secondary outcomes (significant differences)
Meiners et al. (2015) [81]	15 (ND)	39	RCT-d 48 w (extension study Meijer et al. 2010)	Rituximab 1 g/15 days (n = 20) Placebo (n = 10) Weeks 0 and 2	ESSDAI: 1st course w 24 ($p = 0.006$) w 48 ($p = 0.009$); 2nd course w 24 ($p = 0.005$) w 48 ($p = 0.028$) Patient GDA: 1st course w 24 ($p = 0.011$) w 48 ($p = 0.037$) MFI-GF: 1st course w 24 ($p = 0.016$); 2nd course w 24 ($p = 0.019$) VAS oral dryness: 1st course w 24 ($p = 0.021$) w 48 ($p = 0.010$); 2nd course w 24 ($p = 0.053$) VAS ocular dryness: 1st course w 24 ($p = 0.041$)	RF (kI U/L): 1st course w 24 ($p = 0.001$) w 48 ($p = 0.003$); 2nd course w 24 ($p = 0.002$) w 48 ($p = 0.056$) IgG (g/L): 1st course w 24 ($p = 0.001$) w 48 ($p = 0.004$); 2nd course w 24 ($p = 0.002$) B-cells (10^9/L): 1st course w 24 ($p = 0.002$) w 48 ($p = 0.011$); 2nd course w 24 ($p = 0.001$) w 48 ($p = 0.002$)

RCT randomized controlled trial, *-d* double-blind, *-s* single-blind, *-c* crossover; *d* day; *g* gram, mo months, *mg* miligrams, *n* number, *w* weeks, *y* years, *NS* no significant differences, *ND* not detailed, *VAS* visual analogue scale, *LISS* lissamine staining, *SWS* stimulated whole salivary flow, *ESR* erythro-sedimentation rate, *CRP* C-reactive protein, *RF* rheumatoid factor, *Igs* serum immunoglobulins, *abs* autoantibodies, *FACIT-F* Functional Assessment of Chronic Illness Therapy, *PROFAD* Fatigue Profile of Fatigue and Discomfort, *MFI* Multidimensional Fatigue Inventory, *SF-36* Medical Outcomes Short Form, *GDA* Global disease activity, *MFI-GF* multidimensional fatigue inventory general fatigue, *ESSDAI* EULAR Sjögren's Syndrome Disease Activity Index, *CI* confidence interval, *OR* odds ratio

lymphoproliferation (monoclonal lymphocytic infiltration, myoepithelial sialadenitis and lymphoma). Other studies have reported abnormal and aberrant BLyS expression in B cells infiltrating the salivary glands [39] and a key role in the formation of ectopic germinal centers in pSS [38]. Raised levels of BLyS also increase the survival of self-reactive B cells and facilitate abnormal tissue infiltration into follicle/marginal zone niches [39].

There are no randomized controlled data on the use of B-cell activating factor (BAFF)-targeted therapies in patients with pSS. The BELISS trial [40] was an open-label study in 30 pSS patients with systemic or early-onset disease treated with 10 mg/kg of belimumab (weeks 0, 2 and 4, and then every four weeks until week 24). The primary endpoint was evaluated at week 28 (improvement in ≥ 2 of the following items: dryness, fatigue, musculoskeletal pain, physician systemic activity and reduction in biomarkers) and was achieved in 18 (60%) patients, with a higher rate of response in patients with early disease (73%) and those with parotid enlargement (77%), with no improvement in two patients with low-grade parotid lymphoma. The ESSDAI score decreased from 8.8 to 5.59 and the European League Against Rheumatism (EULAR) Sjögren's Syndrome Patient Reported Index (ESSPRI) score from 6.44 to 5.56. With respect to adverse events, one patient developed pneumococcus meningitis. Treatment with belimumab restored B-cell subset subpopulations and normalized BAFF-R expression after 24 weeks [41]. An extended analysis after 52 weeks of therapy recently reported that, at week 28, 13 (87%) of the 15 responders maintained the response, the improvement in ESSDAI (especially of the glandular, lymphadenopathy and articular domains) and the reduction in B-cell biomarkers, while diagnostic tests (salivary flow, Schirmer test, focus score) did not change [42]. A recent study has reported the follow-up of the 13 responders after the end of belimumab treatment, and found an increase in the ESSDAI score, rheumatoid factor (RF) levels, immunoglobulin (Ig)M levels and serum BLyS levels [43].

Other BAFF-targeting agents are under investigation in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), including the recombinant glycoprotein atacicept (TACI-Ig), blisibimod (which binds to both cell membrane-expressed and soluble BAFF) and tabalumab (a human IgG4 monoclonal antibody that binds and neutralizes both membrane and soluble forms of BAFF) (Fig. 1) [44]. Development of briobacept (BAFF receptor BR3-Fc fusion protein) appears to have been discontinued. VAY736 is a monoclonal antibody targeting the BAFF receptor and is currently undergoing evaluation in pSS in a phase 2 clinical study (NCT02149420; estimated study completion June 2017).
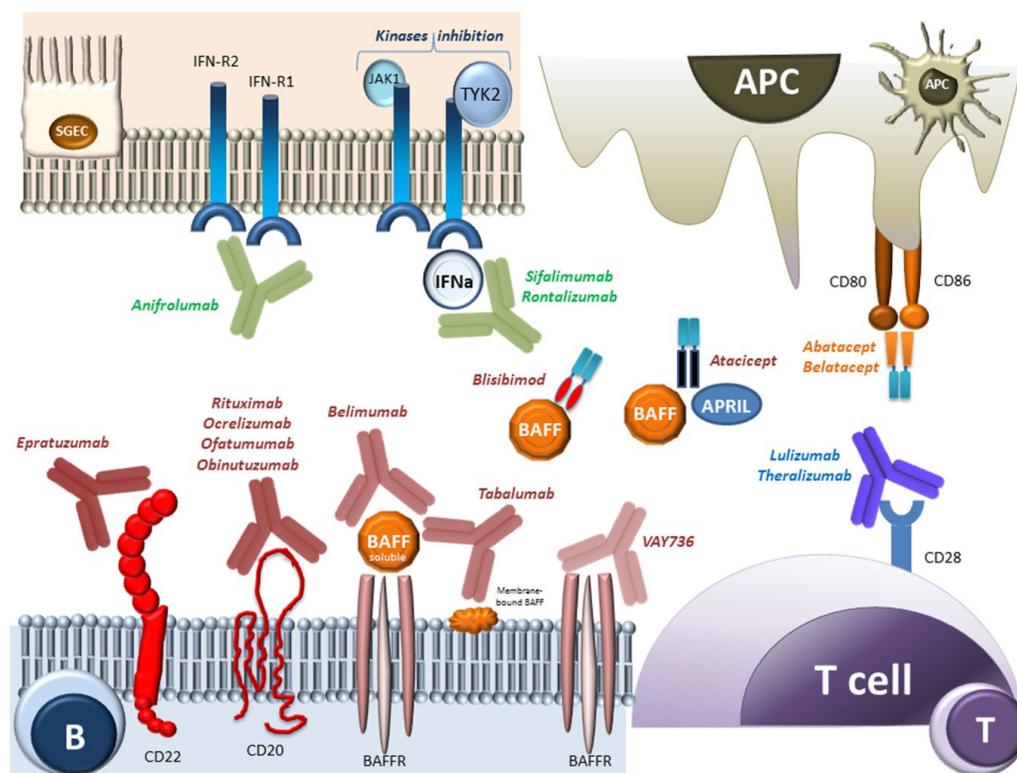
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Fig. 1 Treating the underlying pathophysiology of primary Sjögren Syndrome: main biological agents and molecular targets (IFN, kinases, BAFF, co-stimulatory molecules). *IFN* interferon, *TYK* tyrosine kinase, *JAK* Janus kinase *SGEG* salivary gland epithelial cell, *BAFF* B-cell activating factor, *APC* antigen presenting cell, *APRIL* a proliferation-inducing ligand (TNF ligand superfamily member 13), *BAFFR* B-cell activating factor receptor

3.2 T-Cell Targeting

There are few studies targeting T cells in pSS (Table 6). Abatacept is a fusion molecule that binds the CD80 and CD86 receptors of the antigen-presenting cells and blocks their specific interaction with the CD28 T cell receptor, inhibiting full T-cell activation and T-cell-dependent B-cell activation (Fig. 1). In 2013, the results of a pilot study analyzing the effects of 8 doses of abatacept in 11 pSS patients [45] showed a reduction in lymphocytic foci (mainly FoxP3+ T cells), an increase in peripheral blood B cells (due to expansion of the naive B-cell pool) and a decrease in gamma-globulin levels. The results of a small open-label study that assessed the efficacy and safety of 8 intravenous abatacept infusions in 15 patients with early and active disease in 2014 [46] showed that ESSDAI, ESSPRI, RF and IgG levels decreased significantly during treatment; fatigue and health-related quality of life

(HRQOL) parameters improved significantly, while salivary and lacrimal gland function did not change. Finally, a recent study in 32 patients with SS associated with RA reported mean simplified disease activity index (SDAI) reductions and increases in Schirmer test scores, with adverse events, mainly infections, being reported in 5 patients (16%) [47].

3.3 Targeting Cytokines

At the beginning of this century, agents targeting cytokines were the first type of biological agents tested in pSS, following the excellent results of tumor necrosis factor (TNF)-targeted therapies in RA, although RCTs showed a lack of efficacy [28] (Table 7). Some recent studies have explored the potential benefits of targeting other cytokines involved in the etiopathogenesis of pSS, including type I IFN (ongoing trial, estimated study completion date August

Table 6 Open-label studies evaluating abatacept in patients with primary Sjögren Syndrome

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (no. of patients)	Outcomes evaluated	Significant differences
Adler et al. (2013) [45]	11 (11)	47	Prospective 108 w	Abatacept IV: a total of 8 weight-adapted doses (500 mg below 60 kg bodyweight or 750 mg above 60 kg bodyweight per infusion) at w 0, 2, 4, 8, 12, 16, 20, 24 ($n = 11$)	Salivary gland biopsy (Histopathologic and immunohistochemical evaluation of labial salivary gland biopsy specimens) Saliva and blood parameters (Saxon test, concentrations of the immunoglobulin isotypes IgG, IgA, and IgM) Serum gamma-globulins, gm/l Peripheral blood cells	Numbers of lymphocytic foci decreased ($p = 0.041$) Numbers of local FoxP3, T cells decreased ($p = 0.037$) Peripheral blood, B cells increased ($p = 0.038$) Expansion of the naive B-cell pool ($p = 0.034$) Total lymphocytes increase ($p = 0.044$) and for CD4 cells ($p = 0.009$) Gamma globulins decreased ($p = 0.005$) Saliva production increased ($p = 0.029$)
Meiners et al. (2014) [46]	15 (12)	43	Prospective 48 w	Abatacept IV (10 mg/kg) on days 1, 15 and 29, and then every 4 w (total treatment period 24 w). ($n = 15$)	ESSDAI ESSPRI Patient's GDA Physician's GDA SWS (mL/min) UWS (mL/min) Parotid saliva, stimulated (mL/min) Schirmer (mm/5 min) TBUT RF (klU/L) IgG (g/L)	ESSDAI ($p < 0.05$) ESSPRI ($p < 0.05$) Patient's GDA ($p < 0.05$) Physician's GDA ($p < 0.05$) RF (klU/L) ($p < 0.05$) IgG (g/L) ($p < 0.05$)
Tsuboi et al. (2015) [47]	32 (32)	55	Prospective 48 w	Abatacept IV (500 mg for patients weighing <60 kg, and 750 mg for those weighing 60 kg) at w 0, 2, 4, and every 4 w, for 1 y ($n = 32$) Corticosteroid ($n = 15$) MTX ($n = 24$)	RA-associated secondary SS Tender and swollen joints Physicians VAS Patients global VAS SDAI, DAS 28 ESR, CRP, RF Patients VAS (dry mouth, dry eye, and parotid pain) Physicians VAS (dry mouth, keratoconjunctivitis sicca, and general condition), Saxon's test Schirmer test Abs, serum IgG level	SDAI at baseline to 24 w ($p < 0.05$) Clinical remission by SDAI (25.8%) at 24 w Saliva volume (assessed by Saxson's test) increased ($n = 29$) Greenspan grading 1/2 of labial salivary glands biopsy, saliva volume increased from 0 to 24w ($n = 11$; $p < 0.05$) Schirmer test increase from 0 w to 24 w ($n = 25$; $p < 0.05$)

mo months, *n* number, *w* weeks, *y* years, *NS* no significant differences, *mg* miligrams, *Kg* kilogram, *IV* intravenous, *VAS* visual analogue scale, *TBUT* break-up time test, *UWS,* unstimulated whole salivary flow rate, *SWS* stimulated whole salivary flow, *ESR* erythrosedimentation rate, *CRP* C-reactive protein, *RF* rheumatoid factor, *Igs* serum immunoglobulins, *abs* autoantibodies, *GA* global assessment, *MFI* Multidimensional Fatigue Inventory, *SF-36* Medical Outcomes Short Form, *ESSPRI* European League Against Rheumatism (EULAR) Sjögren's Syndrome Patient Reported Index, *ESSDAI* EULAR Sjögren's Syndrome Disease Activity Index, *GDA* global disease activity, *MTX* methotrexate, *RA* rheumatoid arthritis, *SS* Sjögren Syndrome, *DAS28* disease activity score, *SDAI* Simplified Disease Activity Index

Table 7 Main studies targeting cytokines in patients with primary Sjögren Syndrome*

Author (y)	N (female)	Mean age (y)	Study design (duration)	Drug (number of patients)	Primary outcome (results)	Secondary outcomes (significant differences)
Mariette et al. (2004) [99]	103 (ND)	54	RCT-d 22 w	Infliximab infusions (5 mg/kg) (n = 54) Placebo (n = 49) Weeks 0, 2, 6	No clinical efficacy at 22 w (joint pain, fatigue, dryness VAS improvement 30% at between weeks 0 and 10) (20.4 vs. 16.7%, p = 0.62)	IgM mg/dL (p = 0.001)
Sankar et al. (2004) [100]	28 (26)	55	RCT-d 12 w	Etanercept 25 mg (n = 14) Placebo (n = 14) Twice-weekly	No clinical efficacy at 12 w	Dry mouth (NS) Dry eyes (NS) Schirmer I test, mm/ 5 min (NS) Van Bijsterveld score (NS) Total stimulated saliva flow (NS) IgG, mg/dL (NS) ESR, mm/h (NS)
Zandbelt et al. (2004) [101]	15 (14)	48	Prospective 12 w	Etanercept 25 mg twice per week (n = 15)	No clinical efficacy at 12 w	Fatigue VAS at w8 (p < 0.05) CRP rates at w 12 (p = 0.05)
Norheim et al. (2012) [48]	26 (19)	55	RCT-d 4 w	Anakinra 100 mg/day w 0, w 4 (n = 13) Placebo (n = 13)	NS difference between the groups in fatigue scores at w 4 50% reduction in fatigue VAS (p = 0.03)	NS difference evaluation of laboratory results
St. Clair et al. (2015) [49]	52 (ND)	ND	RCT-d 24 w	Baminercept subcutaneous injections of 100 mg every week (n = 33) Placebo (n = 19)	No more effective than placebo in increasing salivary flow (0.01 vs. 0.06 mL/min; p = 0.37) or reducing ocular dryness	Was associated with improvement in the ESSDAI (−1.6 vs. −0.25; p = 0.043)

RCT randomized controlled trial, *-d* double-blind, *mg* milligram, *w* weeks, *y* years, *min* minute, *VAS* visual analogue scale, *ESR* erythrosedimentation rate, *CRP* C-reactive protein, *Igs* serum immunoglobulins, *NS* no significant differences, *ESSDAI* EULAR Sjögren's Syndrome Disease Activity Index

* Two small open-label studies reporting therapeutic benefits of the use of infliximab in primary SS patients published in 2001 and 2002 were retracted by the authors in 2013

2018) (Fig. 1), IL-1 (no significant results in a small RCT) [48] and IL-6 (a phase III RCT is actively recruiting patients, estimated study completion date March 2017).

Baminercept inhibits the lymphotoxin pathway, which is important in lymphoid tissue organization and chronic inflammation. St. Clair et al. [49], randomized subjects in a 2:1 ratio to receive 24-weekly subcutaneous injections of baminercept 100 mg or placebo. The primary endpoint was the change in pilocarpine-stimulated whole salivary flow (SWSF) between baseline and week 24. The results showed that baminercept therapy was no more effective than placebo in increasing salivary flow or reducing ocular dryness, and was accompanied by an imbalance in transaminase elevations and two cases of reversible grade 3 hepatic injury. The finding that baminercept therapy was associated with an improvement in the ESSDAI warrants further study.

3.4 Targeting Intracellular Pathways

New biological agents targeting intracellular pathways in solid and hematological neoplasia are now being investigated in autoimmune diseases. Most cytokines signal through the JAK/STAT pathway; the cytokine receptor on the cell surface is a protein that forms a stable association with a cytoplasmic tyrosine kinase known as a Janus kinase (JAK). The JAK family comprises four members (JAK1, JAK2, JAK 3 and Tyk2) and their activation subsequently phosphorylates additional targets, including the receptors and the major substrates of STATs (latent cytoplasmic transcription factors) [50]. Selective and non-selective JAK inhibitors have already been tested in some autoimmune diseases (psoriasis, inflammatory bowel disease, RA) and trials are underway in patients with SLE and SS

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(NTC02610543 estimated study completion date May 2020) (Fig. 1).

Cathepsin S is a lysosomal protease involved in the degradation of damaged or unnecessary proteins by the endolysosomal pathway. It also acts as a major histocompatibility complex (MHC) class II antigen presentation molecule and has been linked with the pathogenesis of arthritis, cancer and cardiovascular disease [51], and cathepsin inhibitors are being investigated in the treatment of SLE and lupus nephritis [52]. In pSS, cathepsin S activity is increased in the lacrimal glands and tears of NOD mice [53]. Hamm-Alvarez et al. [54] recently reported that median tear cathepsin S activity in patients with pSS was four-fold higher than in patients with other autoimmune diseases, two-fold higher than in patients with nonspecific dry eye disease, and 41-fold higher than in healthy control subjects. A trial is underway in SS patients (NCT02701985, estimated study completion date June 2017).

The ubiquitin/proteasome system (UPS) plays a key role in modulating numerous cellular proteins to regulate cellular processes such as signal transduction, growth, proliferation, differentiation and apoptosis. The use of proteasome inhibitors such as bortezomib has revolutionized the treatment of B-cell lineage neoplasias [55]. Jakez-Ocampo et al. [56] reported the successful use of bortezomib in a pSS patient with systemic involvement who was refractory to conventional treatment, with a significant clinical response (improvement in fatigue, reduction in hypergammaglobulinemia and serum viscosity, and disappearance of hyperglobulinemic purpura).

4 Future Prospects

4.1 Improving the Design of RCTs in Primary SS

Several small RCTs in pSS patients have shown promising results on the use of very different therapeutic agents. However, the results of the largest trials (>100 patients included) have, until now, shown no significant results for the primary outcomes between the interventional and the placebo arms, even though the drugs tested (infliximab, rituximab and hydroxychloroquine) differ. Why is this?

Dryness of the mucosal surfaces is the pivotal, but not the only, clinical involvement that characterizes pSS. This has strongly influenced how the diagnosis of pSS is made, since the classification criteria currently used (AECG) only evaluate glandular involvement and fail to capture the full spectrum of pSS involvement. In addition to dryness, chronic pain and fatigue complete the clinical triad with the strongest influence on the dramatic reduction in HRQOL of pSS patients. Therefore, large trials chose subjective evaluation (VAS) of these symptoms (dryness, pain and fatigue) as the main primary outcomes. These symptoms affect more than 90% of patients with pSS but, unfortunately, are among those with a less-specific nature. The considerable influence of personal and environmental factors on the intensity of this triad of symptoms could explain the lack of significant differences between therapeutic and placebo arms in larger trials. In a recent study, Milin et al. [57] evaluated 95 patients with sicca syndrome (55 had pSS): the pSS group had a significantly higher proportion of patients with abnormal objective tests for dryness although the degree of altered HRQOL [SF-36, HADS, and Multidimensional Fatigue Inventory (MFI) scores] was similar in the two groups, with anxiety being more common than depression in both groups. Systemic involvement was not a major determinant of HRQOL changes in patients with pSS, and the authors concluded that sicca syndrome was associated with severe alterations in HRQOL regardless of the fulfillment or not of pSS criteria. Cornec et al. [58] found that SF-36 scores indicated marked HRQOL impairments in their population with active pSS, and the factors most strongly associated with HRQOL impairment were ESSPRI patient-reported symptoms, with pain and ocular dryness intensity showing independent associations with HRQOL, while Lee et al. [59] found that the HRQOL of pSS patients was significantly lower than that of the general population, and that the ESSPRI was an independent predictor of HRQOL in pSS patients.

Another key point is the inclusion and exclusion criteria applied in RCTs, which often select non-representative subsets of real-life pSS patients [60]. Oni et al. [61] calculated the percentage of participants in the UK Primary Sjögren's Syndrome Registry (UKPSSR) who would fulfill eligibility criteria for previous/current clinical trials in pSS, and found figures ranging from 75 to 14%. The recently proposed definitions of disease activity levels (ESSDAI, clinESSDAI, DAS, organ involvement definitions) by the EULAR-SS Group has provided a useful and objective tool for the assessment of systemic involvement in pSS [62–65]. In addition, it is essential to determine what kind of side effects appeared in previous studies (Table 8) in order to improve the design of future trials involving similar etiopathogenic pathways.

4.2 Searching for Predictive Factors of Biological Response

Efforts should be made to introduce personalized treatment in pSS. In the last two years, several studies have tried to identify predictive factors of response to B-cell targeted therapies. Delli et al. reported that a high pretreatment number of CD20+ B cells/mm(2) in the parotid gland parenchyma predicts a better responsive to rituximab [66],

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Table 8 Adverse events reported in controlled and uncontrolled studies using biological therapies in patients with primary Sjögren Syndrome

Author (y)	N (female)	Study design (duration)	Drug (number of patients)	Related to infusion	Infection	Cancer	Others
Gottenberg et al. (2005) [89]	6 (6)	Retrospective (8 mo) (32 w)	Rituximab 375 mg/m² W 0, 1, 2, 3 ($n = 5$) W 0,1 ($n = 1$)	Infusion related ($n = 1$) Serum sickness ($n = 1$)	–	–	–
Pijpe et al. (2005) [83]	15 (14)	Prospective 12 w	Rituximab 375 mg/m² W 0, 1, 2, 3 ($n = 15$)	Infusion-related ($n = 2$) Serum sickness ($n = 3$), all HACA+	Herpes zoster ($n = 1$)	–	HACAs: 4/8 of early SS, 0/7 in the other group
Devauchelle-Pensec et al. (2007) [84]	16 (14)	Prospective 36 w	Rituximab 375 mg/m² W 0, 1 ($n = 16$)	Infusion-related ($n = 2$) Delayed reactions ($n = 8$) Serum sickness ($n = 4$)	–	Lymphoma ($n = 1$)	–
Seror et al. (2007) [90]	16 (16)	Retrospective 14.5 mo (56 w)	Rituximab 375 mg/m² W 0, 1, 2, 3 ($n = 14$) Other regimens ($n = 2$)	Serum sickness ($n = 2$)	Herpes ($n = 1$)	–	HACAs in 1/8 One patient with PN-cryo worsened
Dass et al. (2008) [79]	17 (ND)	RCT-d 6 mo (24 w)	Rituximab 1 g/15 days ($n = 8$) W 0 and 2	Infusion related ($n = 2$) Serum sickness ($n = 1$)	Gastroenteritis ($n = 1$)	–	–
Meijer et al. (2010) [80]	30 (29)	RCT-d 48 w	Rituximab 1 g/15 days ($n = 20$) W 0 and 2	Infusion related ($n = 4$) Serum sickness ($n = 1$)	Otitis ($n = 2$) Upper respiratory ($n = 4$) Ocular toxoplasm ($n = 1$) Parotid gland ($n = 3$)	–	–
Ramos-Casals et al. (2010) [92]	15 (15)	Retrospective (ND)	Rituximab 375 mg/m² W 0, 1, 2, 3 ($n = 11$) Rituximab 1 g W 0, 2 ($n = 4$)	–	Urinary infection ($n = 1$)	–	Interstitial pneumonitis ($n = 1$)
Tony et al. (2011) [93]	4 (ND)	Retrospective (ND)	Rituximab ($n = 4$)	Infusion-related ($n = 1$)	–	–	–
Mekinian et al. (2012) [86]	17 (14)	Prospective 33 mo (132 w)	Rituximab 1 g/15 days W 0, 2 ($n = 17$)	Infusion-related ($n = 1$)	Severe cutaneous infection ($n = 1$) CMV infection ($n = 1$)	–	Mild arterial hypertension episodes ($n = 2$)

Table 8 continued

Author (y)	N (female)	Study design (duration)	Drug (number of patients)	Related to infusion	Infection	Cancer	Others
St. Clair et al. (2013) [88]	12 (12)	Prospective 52 w	Rituximab 1 g/15 days W 0, 2 (n = 12)	Severe AE reaction to pneumococcal vaccine (n = 1); non-severe (n = 2)	–	Squamous cell carcinoma (+301 days)	–
Carubbi et al. (2013) [87]	41 (40)	Prospective 120 w	Rituximab 1 g/15 days Weeks 0, 2 (n = 41) Every 24 w (n = 19)	–	–	–	
Gottenberg et al. (2013) [94]	78 (ND)	Retrospective (ND)	Rituximab 375 mg/m² Weeks 0, 1, 2, 3 (n = 11) Rituximab 1 g Weeks 0, 2 (n = 67)	Immediate infusion reactions (severe n = 3) Delayed serum sickness-like (severe n = 1)	Pulmonary infection (n = 1) CMV lung infection (n = 1) SA lung infection (n = 1)	Squamous cell carcinoma (n = 1) Paget's cancer of the nipple (n = 1)	Hypogamma (n = 4)
Devauchelle-Pensec et al. (2014) [29]	122 (97)	RCT-d 24 w	Rituximab 1 g/15 days (n = 63) Week 0 and 2	Infusion related (n = 7)	Bronchitis, urinary and cutaneous infections (n = 35)	Squamous cell carcinoma of the skin (n = 1) Breast cancer (n = 1)	Purpura (n = 1) Cytopenia (n = 1) Hyperglycemia (n = 1)
Bowman et al. (2015) [82]	133 (70)	RCT-d 48 w	Rituximab 1 g/15 days (n = 67) Placebo (n = 66) Weeks 0, 2, 24, 26	Infusion related (n = 1)	–	–	Serious adverse events (n = 10)
Jiang et al. (2015) [31]	6 (6)	Retrospective (ND)	Rituximab 200 mg x1 (n = 1) Rituximab 100 mg x1, weeks 0, 1 (n = 1) Rituximab 200 mg x1, weeks 0, 1 (n = 1) Rituximab 500 mg x1, weeks 0, 1 (n = 3)	–	–	Lymphoma (n = 1)	–
Steinfeld et al. (2006) [34]	16 (14)	Prospective 18 w	Epratuzumab (360 mg/m²) at 0, 2, 4, and 6 w (n = 15)	Severe infusion related (n = 1) (discontinued at 1st infusion) Moderate grade-3 acute infusion reaction (n = 1). Discontinued at 3rd infusion Mild infusion related (n = 2)	Sinusitis (n = 1) Dental abscess (n = 1)	–	Transient ischemic attack with secondary seizure (n = 1) Osteoporotic fracture (n = 1) Headache, paresthesia (n = 3) Fever, palpitation, bone pain, carpal tunnel syndrome, diarrhea, and dyspepsia (ND)

Table 8 continued

Author (y)	N (female)	Study design (duration)	Drug (number of patients)	Related to infusion	Infection	Cancer	Others
Mariette et al. (2015) [40]	30 (30)	Prospective 24 w	Belimumab, 10 mg/kg, Weeks 0, 2, 4 and then every 4 w to w 24 ($n = 30$)	–	Pneumococcal meningitis ($n = 1$) Pneumonia ($n = 1$) Sinusitis ($n = 1$) Rhinitis/pharyngitis ($n = 7$) Bronchitis ($n = 1$) Herpes labialis ($n = 1$) Urinary tract infection ($n = 2$) Gastroenteritis/diarrhea ($n = 2$)	Breast cancer ($n = 1$)	Scleroderma ($n = 1$) Headache ($n = 9$) Neutropenia ($n = 5$) Oral aphthosis ($n = 1$)
De Vita et al. (2015) [42]	19 (19)	Prospective 52 w	Belimumab, 10 mg/kg, w 0, 2, 4 and then every 4 w to w 52 ($n = 19$)	–	Rhinopharyngitis ($n = 2$) Gastroenteritis ($n = 1$) Urinary tract infection ($n = 1$) Pneumonia ($n = 1$) Vaginal fungal infection ($n = 1$) Non-complicated cutaneous infection ($n = 1$)	–	Headache at the end of the infusion ($n = 1$) Mild transient neutropenia ($n = 2$)
Mariette et al. (2004) [99]	103 (ND)	RCT-d 22 w	Infliximab infusions (5 mg/kg) ($n = 54$) Weeks 0, 2, 6	Infusion related ($n = 2$) Isolated cutaneous facial eruption ($n = 1$)	Pneumococcal septicemia ($n = 1$)	Breast cancer ($n = 1$)	Autoimmune hepatitis ($n = 1$)
Sankar et al. (2004) [100]	28 (26)	RCT-d 12 w	Etanercept 25 mg week Twice weekly ($n = 14$)	Injection-site reaction ($n = 2$)		–	–
Zandbelt et al. (2004) [101]	15 (14)	Prospective 12 w	Etanercept 25 mg Twice weekly ($n = 15$)	–		–	–
Adler et al. (2013) [45]	11 (11)	Prospective 108 w	Abatacept IV: (500 mg below 60 kg bodyweight or 750 mg above 60 kg bodyweight per infusion) at w 0, 2, 4, 8, 12, 16, 20, 24 ($n = 11$)	Lupus-like skin lesions ($n = 1$)	Diverticulitis ($n = 1$)	–	Transient increase in liver enzymes (concomitant rifampin medication for latent tuberculosis) ($n = 1$)

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Table 8 continued

Author (y)	N (female)	Study design (duration)	Drug (number of patients)	Related to infusion	Infection	Cancer	Others
Tsuboi et al. (2015) [47]	32 (32)	Prospective 48 w	Abatacept (500 mg for patients weighing <60 kg, and 750 mg for those weighing 60 kg) IV at w 0, 2, 4, and every 4 w, for 1 y (n = 32); Corticosteroid (n = 15); MTX (n = 24)	–	Urinary tract infection (n = 1); Infected corneal ulceration (n = 1); Bronchitis (n =1)	–	Compression fracture of lumber spine (n = 1); Vomit and diarrhea (n = 1)
Norheim et al. (2012) [48]	26 (19)	RCT-d 4 w	Anakinra 100 mg/day w 0, w 4 (n = 13)	Severe injection site reaction (n = 1) (discontinued); Mild injection site reactions (n = 7)	Gastroenteritis (n = 1)	–	Neutropenia (n = 1)
St. Clair et al. (2015) [49]	52 (ND)	RCT-d 24 w	Baminercept subcutaneous injections of 100 mg every week (n = 33)	–	–	–	SAE (baminercept group) n = 2 with grade 3 hepatic injury who recovered without sequela; Transaminase abnormalities (>ULN) baminercept group [n = 10, (30%) 15 events], placebo group [3 subjects (16%), 5 events]

RCT randomized controlled trial, *-d* double-blind, *g* gram, *m²* square meter, *mo* months, *mg* milligram, *n* number, *w* weeks, *y* year(s), *ND* not detailed, *IV* intravenous, *CMV* cytomegalovirus, *PN-cryo* cryoglobulinemic polyneuropathy, *HACA* human antichimeric antibodies, *hypogamma* hypogamma-globulinemia, *SA Staphylococcus aureus*, *AE* adverse events, *SAE* serious adverse event

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while Cornec et al. [67] found that blood and salivary-gland BAFF-driven B-cell hyperactivity was associated with rituximab inefficacy in pSS. Baseline serum BAFF levels correlated with the proportion of salivary gland B-cells and other B-cell-activation markers and was associated with the clinical response, with higher levels in non-responders. In pSS, half of the patients display intense BAFF-driven B-cell activation and do not respond to a single course of rituximab. The duration of B-cell depletion was not associated with the clinical response, but responders had lower baseline proportions of salivary gland B cells. With respect to the prediction of the therapeutic response to belimumab, the results of the BELISS study showed that low numbers of blood and salivary natural killer cells were associated with a better response to belimumab in pSS. The authors suggest that two distinct subsets of pSS may exist: one with a predominant type I interferon (IFN)-BAFF-B-cell axis (good responders) and one with a predominant type II IFN-NK cell axis (non-responders) [68].

4.3 Intra-Lesional and Sequential use of Biological Therapies

In light of the use of intra-articular anti-TNF agents in RA [69], it seems reasonable to suggest similar approaches in pSS. Demirci et al. [70] recently reported the use of intra-lesional rituximab in a 41-year-old women with pSS presenting with mucosa-associated lymphoid tissue (MALT) lymphoma of the lacrimal gland with an initial complete response to intravenous rituximab but who had a recurrence Eleven months previously; intra-lesional rituximab (50 mg/mL) was injected into the left lacrimal gland, followed by injection in the right lacrimal gland seven months later. There was a significant decrease in the size of the bilateral lacrimal glands and subjective improvement in dry-eye symptoms after a follow-up of 23 months.

The combined blockade of different molecular targets in a simultaneous or sequential strategy is used in patients with neoplasia. These strategies are now under investigation in autoimmune diseases. Some patients with SLE may present with post-rituximab SLE flares associated with raised circulating BAFF levels, and a high proportion of plasmablasts in the B-cell pool. BAFF not only increases the survival of autoreactive B cells (including plasmablasts), but also stimulates Tfh cells. Repeated rituximab infusions can result in a feedback loop characterized by ever-rising BAFF levels, surges in autoantibody production, and worsening of disease. As suggested by this hypothesis, some authors are now proposing the use of BAFF-blocking agents after the use of B-cell depleting agents [71], and a recent successful use of this sequential

regimen has been reported in a patient with refractory lupus nephritis [72].

In pSS, De Vita reported [73] sequential therapy with belimumab followed by rituximab in a patient with severe, refractory parotid low-grade B-cell MALT lymphoma and cryoglobulinemic vasculitis. The patient presented complete, persistent regression of lymphoma and healing of a refractory skin ulcer, and rituximab was administered as maintenance therapy 6 and 12 months later. No significant side effects were noted, except for a marked decrease in serum IgM. RF and cryoglobulins became persistently negative and serum BAFF and C4 levels normal, with a total follow-up of nearly four years. De Vita et al. hypothesized that the overexpression of BAFF in mucosa-associated lymphoid tissue may decrease the efficacy of rituximab. One clinical trial of subcutaneous belimumab and intravenous rituximab co-administration is underway (NCT02631538). This is a multicenter, double-blind (sponsor open), randomized, placebo-controlled trial in subjects with active pSS designed to understand the safety and tolerability profile of belimumab/rituximab co-administration and of belimumab monotherapy, and to evaluate whether either co-administration therapy or belimumab monotherapy has a substantive effect on disease activity. The total participation time of a subject in this study will be approximately up to a maximum of 2 years (estimated study completion date October 2019).

4.4 Gene Therapy: the Future?

Gene therapy is an emerging therapeutic field in complex human diseases. It consists of introducing specific genetic material into target cells to compensate for abnormal genes (reparative medicine) or to make a beneficial protein (pharmacological purpose) without producing toxic effects on surrounding tissue [74, 75]. Gene delivery systems can be divided to two major types: vector-based (viral or non-viral vector) and physical delivery technologies (electroporation, gene gun, ultrasound). A relatively new physical delivery technology for gene delivery consists of microneedles (metal, coated or dissolving) [76], and future studies will employ ultrasound-assisted and pseudotyped adeno-associated viral vector-mediated gene transfer [77].

A very recent study has initiated investigation of the potential use of gene therapy in pSS. Lai et al. [78] reported the successful use of gene therapy in a murine model, in which increased gland permeability was achieved by regulating the influence of a specific cytokine (bone morphogenetic protein 6) on the expression of aquaporin 5, a water channel critical for salivary gland fluid secretion. Therapy not only resulted in the restoration of secretory gland function but also resolved the hallmark salivary gland inflammation.

5 Conclusions

New therapeutic approaches for pSS are under development. This century has seen the inclusion of a new group of drugs (biological agents) in the management of pSS. Unfortunately, the results of the few controlled trials carried out are disappointing, especially because the agents tested (overwhelmingly directed against B cells) can be considered as reasonable targets from the etiopathogenic point of view. The designs of future trials in pSS should be rethought in order to select patients closer to those seen in clinical practice. GWAS aiming at better clinical characterization of pSS in large, worldwide populations of patients with pSS may be key to designing future studies. Meanwhile, ongoing RCTs in pSS are shifting from targeting B cells to targeting other etiopathogenic pathways involving T cells, cytokines, intracellular mechanisms or gene therapies. We may be looking forward to a disruptive period in the therapeutic approach to this frequent, complex systemic autoimmune disease.

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