

# Sjogren's Syndrome: A Clinical and Biochemical Analysis

by

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# **Abstract**

## **Introduction**

Sjogren's syndrome (SS) is a systemic autoimmune disease that presents to eye care practitioners with the hallmark symptom of "dry eye." Stratifying dry eye patients as Sjogren's positive or negative is a critical differential diagnosis, as SS patients have numerous systemic complications and a forty times greater risk of developing lymphoma. As such, management of this relatively common dry eye sub-population requires specialized care. Since a firm diagnosis requires testing that is both invasive and expensive, patients should be protected from these tests if they are not warranted.

In this thesis, studies were therefore undertaken to determine if SS dry eye could be differentiated from other forms of dry eye using two methods: 1) standard clinical tests used in a multi-disciplinary Sjogren's syndrome clinic and 2) subsequent biological evaluation of collected tear samples and cells from the ocular surface. The former would allow eye care practitioners to conduct appropriate tests and pose suitable questions to differentiate these subgroups, and the latter might serve in the future as a relatively non-invasive quantitative means of differentiating such groups through biomarkers.

The specific aims of each chapter were as follows:

- Chapter 1. To introduce the concepts addressed by this body of work.
- Chapter 2. To provide a review of the literature on Sjogren's syndrome.

- Chapter 3. To conduct a retrospective analysis of an existing database of SS and aqueous deficient dry eye (keratoconjunctivitis sicca, KCS) patients to determine those clinical tests and historical answers that differentiate SS patients from those with KCS alone.
- Chapter 4. To quantify and compare the concentrations of total protein, lysozyme and lipocalin in the tears of SS subjects, KCS subjects and control (non-dry eye, NDE) subjects.
- Chapter 5. To quantify and compare the tear concentration of soluble MUC16, the concentration of membrane bound MUC16 and the expression of conjunctival epithelial MUC16 mRNA in SS subjects, KCS subjects and NDE control subjects.
- Chapter 6. To summarize the results of this thesis and to discuss future research studies.

## **Methods**

- Chapter 3: The records of all patients seen at the Toronto Western Hospital Sjogren's Syndrome Clinic from October 1992 to July 2006 were reviewed and documented (n=420). Patients were diagnosed as primary SS (pSS) or secondary SS (sSS) by the American European consensus criteria (AECC) of 2002. Those patients seen at the clinic who were not diagnosed with SS and who had symptoms of dry eye and Schirmer scores of  $\leq 10$  mm in 5 minutes in at least one eye were included as dry eye controls. Factor analysis was performed followed by recursive partitioning. Classification trees were developed that demonstrated which characteristics best distinguished SS from KCS.
- Chapter 4: Three subject groups were entered into this study: SS, KCS and NDE controls (n=25 in each group). Tears were collected using an "ocular wash" method. These

washes were pooled and the DC Protein Assay Kit® (BioRad) was used to calculate total tear film protein. Concentrations of tear lipocalin and lysozyme were determined via Western blotting.

- Chapter 5: As in chapter 4, samples were collected from 3 groups of subjects: SS, KCS and NDE controls. Tears were collected using an “ocular wash” technique. Conjunctival epithelial cells were collected via impression cytology from each subject. MUC16 protein or mRNA was extracted from the conjunctival epithelial cells. All total protein determinations were conducted using the DC Protein Assay Kit® (BioRad), following the manufacturer’s instructions. Soluble and membrane bound MUC16 was quantified by Western blotting. MUC16 mRNA was measured using real time PCR.

## **Results**

- Chapter 3: Corneal fluorescein and conjunctival rose bengal staining act as independent variables in SS. The observation of rose bengal staining of the temporal conjunctiva is the most important non-invasive test in identifying SS and in differentiating SS from KCS disease.
- Chapter 4: SS subjects distinguished themselves by demonstrating significantly lower ( $p < 0.001$ ) total protein concentration ( $0.4 \pm 0.32 \mu\text{g}/\mu\text{L}$ ) compared with the KCS group ( $1.23 \pm 0.72 \mu\text{g}/\mu\text{L}$ ) and NDE group ( $1.21 \pm 0.63 \mu\text{g}/\mu\text{L}$ ). No difference in tear total protein concentration was found between the KCS and NDE groups ( $p = 0.92$ ).

The SS subjects were different in that they displayed a significantly reduced ( $p < 0.0001$ ) lipocalin concentration ( $0.067 \pm 0.05 \text{ AU}$ ) compared with both KCS ( $0.13 \pm 0.05 \text{ AU}$ )

and NDE ( $0.15 \pm 155 \ 0.05$  AU) groups. No difference in tear film lipocalin was found between KCS and NDE groups ( $p=0.19$ ).

No difference ( $p=0.80$ ) was found between any of the groups with respect to the concentration of tear film lysozyme.

- Chapter 5: The SS group differentiated itself by demonstrating significantly higher tear concentrations of soluble MUC16 ( $7.28 \pm 3.97$ ) compared with both KCS ( $3.35 \pm 4.54$ ;  $p=0.004$ ) and NDE ( $1.61 \pm 1.22$ ;  $p<0.0001$ ) groups. The SS group also demonstrated a significantly higher concentration of MUC16 mRNA ( $4.66 \pm 5.06$ ) compared with both KCS ( $1.84 \pm 2.26$ ;  $p=0.01$ ) and NDE ( $1.52 \pm 1.04$ ;  $p=0.003$ ) groups.

No differences in the concentrations of soluble MUC16 and MUC16 mRNA were found between the KCS and NDE groups ( $p>0.05$ ). No difference in membrane bound MUC16 was found between any groups ( $p>0.05$ ).

## **Conclusions**

- Chapter 3: Eye care practitioners can best capture and classify various forms of dry eye through observations of rose bengal staining of the ocular surface.
- Chapter 4: SS patients have lower total protein and lipocalin concentrations in their tears than do NDE control and KCS subjects. All groups have a similar amount of lysozyme.
- Chapter 5: SS patients have increased levels of soluble MUC16 and MUC16 mRNA compared with KCS and control subjects. All groups express similar amounts of membrane bound MUC16.

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## **Dedication**

To all of the patients who suffer from Sjogren's syndrome.

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## List of Symbols and Abbreviations

µg	micrograms
µl	microlitre
AD	aqueous deficiency
AECC	American European consensus criteria
AI	autoimmune
AIDS	acquired immune deficiency syndrome
AMA	anti-mitochondrial antibody
ANA	anti-nuclear antibody
Anti-Mic	anti-mitochondrial antibody
APC	antigen presenting cell
APRIL	a proliferation inducing ligand
ATA	anti-thyroid antibody
AU	arbitrary units
BAFF	B cell activating factor
Bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CIC	conjunctival impression cytology
CREST	calcinosis, Raynaud's, esophageal, sclerodactyly, telangiectasia
CsCl	cesium chloride
CT	connective tissue

ddH <sub>2</sub> O	double distilled water
DE	dry eye
DES	dry eye syndrome
DEWS	dry eye workshop
DMF	dentate missing filled
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
Dscore	dental score
DTT	Dithiothreitol
E	evaporation
EB	extraction buffer
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetra acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	germinal centre
GLB	gel loading buffer
H&E	hematoxylin and eosin stain
HCLE	human cornea-limbal epithelial cell line
HEP	heparin
HEV	high endothelial venules

HIV	human immunodeficiency virus
HLA	human leukocyte-associated
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
INF	interferon
JRA	juvenile rheumatoid arthritis
KCl	potassium chloride
KCS	keratoconjunctivitis sicca
KDa	KiloDalton   wavelength
L	litre
MALT	mucosa-associated lymphoid tumor
mg	milligram
MHC	major histocompatibility complex
ml	millilitre
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
mOsm/Kg	milli osmoles/kilogram
MP	millipore
mRNA	messenger ribo nucleic acid
MUC	mucin

Mw	molecular weight
NaCl	sodium chloride
NDE	non dry eye
ng	nanogram
nm	nanometre
NS	not significant
OCP	ocular cicatricial pemphigoid
PAS	periodic acid-Schiff stain
PBC	primary biliary sclerosis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 0.05% Tween® 20
PCR	polymerase chain reaction
PES	poly ether sulphone
pg	picogram
PI	protease inhibitor
pSS	primary Sjogren's syndrome
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
R2	correlation coefficient
RA	rheumatoid arthritis
RB	rose bengal
rcf	relative centrifugal force

RF	rheumatoid factor
Rn	normalized reporter dye fluorescence
RN	Rneasy™ Mini kit
RNA	ribonucleic acid
RNAse	ribonuclease
RT-PCR	reverse transcription - polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sIgA	secretory immunoglobulin A
SLE	systemic lupus erythematosus
SLK	superior limbic keratoconjunctivitis
SMA	smooth muscle antibody
SS	Sjogren's syndrome
SSA	Sjogren's syndrome protein A
SSB	Sjogren's syndrome protein B
sSS	secondary Sjogren's syndrome
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.05% Tween® 20
TEM	transmission electron microscopy
Th T	helper cell
TNF	tumor necrosis factor

Treg	T regulatory cell
Tris	(hydroxymethyl) aminomethane
WB	Western blotting
WBC	white blood cell

# 1 Introduction

Sjogren's syndrome (SS) is one of the most common systemic rheumatic autoimmune diseases, second only to rheumatoid arthritis in its prevalence in North America.<sup>1</sup> However, the diagnosis remains difficult as the clinical presentation of this disease can be elusively benign. The disease can exist for a long period in subclinical form,<sup>2, 3</sup> making the disease duration of most patients uncertain. These factors contribute to the clinical reality that diagnosis and subsequent treatment may be delayed for many years.<sup>4, 5</sup>

The importance of diagnosing Sjogren's syndrome cannot be overestimated. Identifying and managing these patients is of great importance, as the spectrum of SS ranges from significant symptoms of dryness that interfere with quality of life to a 40 times greater risk for mucus associated lymphoma tumours (MALT).<sup>6</sup> In addition to the ocular manifestations of SS, various systemic complications must also be appropriately managed.

Eye care practitioners are in a unique situation to be involved in the diagnosis of SS, in that SS patients will often present for the first time in their offices with symptoms of dry eyes. Their role is to determine which of these dry eye patients should be sent on for further invasive testing to confirm a diagnosis of SS. The confirmed diagnosis requires blood analysis and a minor salivary gland biopsy, both of which are invasive and expensive.<sup>7</sup> Their role is to stratify these dry eye patients as accurately as possible to improve the sensitivity and specificity of their referrals.

Since the eye is frequently involved in the pathology of SS, it may prove to be a source of biomarkers for this disease. As tear sampling and impression cytology of the



conjunctival tissue are rather benign tests, it is valuable to determine if potential biomarkers could be derived from biological analysis of these ocular samples. The concept of a future in which routine dry eye testing such as history taking, Schirmer and surface staining tests could be followed by relatively simple in-office tear and cell analysis that would allow clinicians to confirm a clear diagnosis of SS without a more invasive workup seems hopeful.

Hence, the hypotheses investigated in this work are as follows:

Sjogren's syndrome can be distinguished from other forms of aqueous deficient dry eye disease:

1. in the eye care clinic through history taking and specific ocular surface testing.
2. in the laboratory through analysis of tear proteins and mucin biomarkers.

This thesis consists of 6 chapters devoted to these topics:

1. Introduction
2. Literature review of Sjogren's syndrome
3. Analysis of a database of SS and KCS patients
4. Analysis of proteins in the tears of SS, KCS and NDE control subjects
5. Analysis of MUC16 concentrations in the tears and surfaces of conjunctival cells and MUC16 mRNA in the conjunctival cells of SS, KCS and NDE control subjects
6. Conclusions and future research.

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## 2 Literature Review

### 2.1 History and Criteria

Sjogren's syndrome (SS) is a chronic systemic rheumatic autoimmune disorder that is characterized by lymphocytic infiltration and malfunction of the exocrine glands, resulting in dry eye and dry mouth. The systemic nature of this disease is confirmed by the presence of autoantibodies in the serum or specific lymphocytic infiltration of the salivary glands. The syndrome can present either alone, in primary Sjogren's syndrome (pSS), or in the context of an underlying rheumatic disease, in secondary Sjogren's syndrome (sSS). Systemic manifestations of the disease may be found in the following systems:

1. Musculoskeletal, including fatigue and fibromyalgia
2. Vascular – Raynaud's phenomenon
3. Cutaneous
4. Pulmonary
5. Gastrointestinal
6. Neurological
7. Lymphoproliferation
8. Gynecological
9. Maternity
10. Renal
11. Hematologic

The systemic rheumatic diseases are a unique group of chronic conditions that include Sjogren's syndrome, systemic lupus erythematosus (SLE), rheumatoid arthritis

(RA), juvenile rheumatoid arthritis, systemic sclerosis, scleroderma and polymyositis/dermatomyositis.<sup>(1)</sup> These diseases are characterized by serum immunoglobins directed at self-antigens that are believed to be responsible for many of the clinical manifestations.

Sjogren's syndrome is distinguished by a 90% female predominance.<sup>(2)</sup> There is some controversy as to the age of onset, as the disease can exist for a long period in subclinical form,<sup>(3, 4)</sup> making the disease duration of most patients uncertain. Some report two age peaks in the diagnosis of this disease: 20-30 years and after menopause to the mid-50s.<sup>(5)</sup> Others state the age of onset as approximately 45 years.<sup>(6-8)</sup> It is known that diagnosis may be delayed for many years.<sup>(6, 8)</sup>

There is a distinct lack of longitudinal studies of SS, and therefore the natural history of the disease is not known. However, it is believed that the disease progresses during the early years after diagnosis.<sup>(6)</sup> Most patients will stabilize at some point, yet some will progress to multi-organ involvement<sup>(6)</sup> and others to malignant lymphoma.<sup>(9)</sup> One study suggests that ocular signs may even improve over the years.<sup>(10)</sup>

### **2.1.1 History**

The history of this disease is chronicled in a 1987 book entitled *Sjogren's Syndrome: Clinical and Immunological Aspects*.<sup>(11)</sup> The first mention of this disease came from a case report at a meeting of the Clinical Society of London in 1888. Hadden described a 63-year-old widow who suffered dry mouth and an insufficiency of lacrimation. Fuchs in 1919 reported on a 54-year-old woman who had dryness of the mouth and eyes and a swollen parotid gland. Gougerot in 1926 grouped three patients together and suggested a syndrome based on dry eyes and dry mouth. The unique

histology of this disease was first described by Albrich in 1928, when he reported a dense lymphocytic infiltration of the lacrimal gland in a patient with bilateral swelling of the parotid glands.

Very early in the descriptions of this sicca complex, an association was made with arthritis. Houwer in 1927 was the first to suggest a relationship of filamentary keratitis and chronic arthritis. There were then several papers published between 1928 and 1933 that described the association between dry eye, dry mouth and arthritis.

Sjogren in 1930 saw a patient with filamentary keratitis, decided to study this disease and published his findings in 1933. It is this work that gave Sjogren's syndrome its name. Over the next decades, many papers described the disease, and it appeared that rheumatoid arthritis was the joint disease most related to this syndrome.

There was, for a time, some confusion of Sjogren's syndrome with the disease described and named by Mikulicz in 1888. His original description was of an individual who presented with swollen lacrimal and submandibular glands who died 6 months after his visit to the clinic. The histology of the glands was described as a "round cell" infiltrate and was assumed to be infectious. The term Mikulicz's disease was then given as a diagnosis to those patients with lymphoma, sarcoidosis, tuberculosis and lead or iodide poisoning who presented with swollen lacrimal and salivary glands. In 1927, Schaffer and Jacobsen defined two main categories of the clinical syndrome described by Mikulicz. One was called Mikulicz's disease, being of unknown etiology and mild clinical course, and the other was called Mikulicz's syndrome, which was caused by a variety of disorders such as leukemia and tuberculosis. Later, Morgan and Castleman in

1953 concluded that Mikulicz's disease and Sjogren's syndrome were histologically identical.<sup>(12, 13)</sup>

It appears that the original descriptions of Sjogren's syndrome were those of secondary SS, as most were associated with rheumatoid arthritis. Also, all signs of dry eye were related to corneal staining and filamentary changes with no description of the conjunctiva.

### **2.1.2 Criteria**

As Sjogren's syndrome became better recognized and described, the diagnostic criteria changed and different sets of criteria were used at different times and in different countries.

The Sjogren-based description of the disease was used through 1965, when Bloch et al.<sup>(7)</sup> described 62 patients in North America with Sjogren's syndrome. The disease was defined as a triad of:

1. keratoconjunctivitis sicca (dry eye)
2. xerostomia (dry mouth) and
3. rheumatoid arthritis or other connective tissue disease.

Bloch et al. included those patients who presented with two of the three components. They excluded specific diseases of the salivary or lacrimal glands such as lymphoma, sarcoidosis and tuberculosis.

The above set of criteria was generally used by clinicians until 1968, when Feltkamp and van Rossum<sup>(14)</sup> and later Fairfax in 1981<sup>(15)</sup> tried to better define the sicca

components of SS. At this time the elements of dry eye and dry mouth were defined as follows:

1. Keratoconjunctivitis (KCS) was probable if symptoms were present OR if the Schirmer scores were positive. KCS was definite if corneal epithelial erosion or filamentary keratitis was present.
2. Xerostomia was probable if certain symptoms were present OR the dorsal tongue showed atrophic changes. Dry mouth was definite if either of the above was present and there was salivary gland enlargement, punctate sialectasis by sialography, greater than 50% reduction in salivary flow rate measurement OR abnormal findings on the salivary gland biopsy.

In 1968 Chisholm and Mason<sup>(16)</sup> created a salivary gland biopsy scale that ranged from class I, which labeled a salivary gland with few lymphocytes, to class IV, which described  $\geq 2$  lymphocyte foci per 4 mm<sup>2</sup>. Greenspan et al. revised this scheme in 1974 by counting foci.<sup>(17)</sup> In this description, a class III infiltrate was described as 1 focus score per 4 mm<sup>2</sup> while a class II infiltrate described scattered lymphoid infiltrates containing  $< 1$  focus per 4 mm<sup>2</sup>.

Whaley et al. in 1973 made minor modifications to the Bloch group definition for the diagnosis of SS in their series of 171 patients.<sup>(18)</sup> They accepted these definitions:

1. Keratoconjunctivitis was present if there was a reduced rate in an anesthetized Schirmer test AND corneal punctate staining or filamentary keratitis, and
2. Xerostomia was present if there was no salivary pooling OR there was salivary gland enlargement.

Daniels et al. in 1975<sup>(19)</sup> defined the salivary component of SS as the presence of focal sialoadenitis in a labial salivary gland biopsy specimen. They required a focus score of greater than 1 focus per 4 mm<sup>2</sup>.

Until 1978 Sjogren's syndrome was generally defined by its association with other connective tissue diseases, particularly rheumatoid arthritis. In 1978 Frost-Larsen et al.<sup>(20)</sup> and in 1979 Moutsopoulos et al.<sup>(21, 22)</sup> introduced the concept of primary and secondary SS. Thus, primary SS (pSS) was present when the patient qualified for diagnosis under the chosen criteria but did not have another connective tissue disease. Secondary SS (sSS) was defined as present when the patients qualified for diagnosis of SS AND had clinical manifestations of another connective tissue disease. This type of distinction continues to this day.

Since that time several groups have focused on better defining the dryness aspects of the disease. However, the road was not smooth and different groups moved toward and away from this rigor. Some adopted more symptom-based clinical definitions and others moved to demand tissue and serum standards.

Moutsopoulos et al. in 1979<sup>(21)</sup> suggested that xerostomia be much more rigorously defined. Decreased parotid flow rate, abnormal parotid scintigraphy findings AND abnormal salivary gland biopsy were required. Dry eye was defined as punctate corneal ulcers AND a reduced Schirmer flow. There was, however, no statement of abnormal numbers for each test.

Manthorpe et al. in 1981<sup>(23)</sup> tried to provide cutoff points for the diagnostic components of dry eye and dry mouth. The criteria were these:



1. Dry eye was present if there were 2 of the following: Schirmer < 10 mm/5 minutes, tear breakup time < 10 seconds, slit lamp corneal staining scores with rose bengal of >4.
2. Xerostomia was present if 2 of the following were present: lymphocytic infiltrates in the labial salivary gland biopsy, unstimulated whole salivary flow of < 1.5 ml/15 min., reduced uptake and secretion in salivary scintigraphy.

Meanwhile, in Scandinavia the Copenhagen criteria<sup>(24)</sup> have been the standard for diagnosing primary SS since 1986. Here pSS is defined as the simultaneous presence of dry eye and xerostomia in patients not fulfilling internationally accepted criteria for other inflammatory connective tissue diseases. In this case the diagnosis of SS depends on clinical observation and does not require a biopsy or serum autoantibodies for inclusion.

The Copenhagen criteria state:

1. Dry eye is present if one of the following is the case: Schirmer-1 test  $\leq$  10 mm/5 min, breakup time  $\leq$  10 sec, rose bengal score  $\geq$  4 (vonBjsterveld criterion adding conjunctiva and cornea). This was the first time that conjunctival staining had been included among the criteria.<sup>(25)</sup>
2. Xerostomia is present if 2 of the following are found: unstimulated whole sialometry  $\leq$  1.5 ml/15 min, abnormal salivary gland scintigraphy, lower lip biopsy score of > 1 focus in 4 mm.

In 1986 a North American Group led by Fox<sup>(26)</sup> used the Daniels salivary gland histology criterion<sup>(27)</sup> (i.e., greater than 1 focus score per 4 mm<sup>2</sup>) and made a major step

in defining the diagnosis of SS. This set of 4 criteria is sometimes referred to as the San Diego Criteria for primary Sjogren's syndrome:

1. dry eye if symptoms AND Schirmer score of <8 mm/5 minutes AND the presence of rose bengal or fluorescein staining of cornea or conjunctiva
2. xerostomia present if symptoms AND decreased basal and stimulated salivary flow rate
3. lymphocytic infiltration of labial salivary gland biopsy (at least 2 foci in 4 mm<sup>2</sup>)
4. presence of rheumatoid factor >1:160, antinuclear antibody >1:160, OR positive SS-A or SS-B antibodies

Definite SS was defined as having all of the above while probable SS was diagnosed without the salivary gland biopsy evidence but with the definite demonstration of decreased salivary function. "Probable SS" remained a part of the literature until the recent American European Consensus Criteria of 2002.<sup>(28)</sup>

More recently, the preliminary European criteria were put forth in 1993 by Vitali et al. These criteria changed the cutoff point for the Schirmer 1 test from 10 to 5 mm in 5 minutes to increase the specificity of the test.<sup>(29)</sup> In this paper secondary SS was defined as being present in those who display characteristic signs of SS as above in addition to clinical features sufficient to allow a diagnosis of RA, SLE, polymyositis, scleroderma or primary biliary cirrhosis (PBC). Exclusions include sarcoidosis, preexisting lymphoma and other causes of dry eye.<sup>(26)</sup>

The San Francisco criteria were published in 1994 by Daniels et al.<sup>(30)</sup> They emphasized the need to have evidence of lymphocytic infiltration of the minor salivary

glands. However, in the same year the European Community Study Group published criteria that did not require serum or histological evidence of the disease.<sup>(31)</sup>

After many meetings and rigid prospective testing of the proposed criteria, the most recent gold standard criteria for diagnosis of Sjogren's syndrome were published as the American European Combined Criteria (AECC) in 2002.<sup>(28)</sup> The criteria are:

1. symptoms of dry eye for at least 3 months
2. symptoms of dry mouth for at least 3 months
3. signs of dry eye: Schirmer I scores of  $\leq 5$  mm in 5 minutes and /or rose bengal or fluorescein staining scores of  $\geq 4/9$  in at least one eye (this scoring is a sum of the cornea, nasal and temporal conjunctiva graded 0-3)
4. signs of dry mouth: salivary flow by unstimulated spitting in a cup of  $\leq 1.5$  ml in 15 minutes (0.1 ml in 1 minute).
5. positive serum findings of autoantibodies to Ro and/or La
6. positive salivary gland biopsy score:  $\geq 1$  focus score in 4 mm of tissue.

A minimum of 4 of these 6 criteria must be met, including one of either serum positive or biopsy positive.

Ocular symptoms are considered to be present if a positive response is given to one of the following:

1. Have you had daily persistent troublesome dry eyes for  $> 3$  months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?

Oral symptoms are considered to be present if there is a positive response to at least one of the following questions:

1. Have you had a daily feeling of dry mouth for >3 months?
2. Have you had recurrent or persistent swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?

The classification of primary and secondary SS is also well defined. In the presence of an associated autoimmune disease (rheumatoid arthritis, systemic lupus erythematosus, scleroderma), secondary SS would be defined as the presence of ocular symptoms OR oral symptoms combined with any two of the following: objective signs of dry eye, objective signs of dry mouth, histopathology.

The exclusion criteria include:

1. past neck and head radiation treatment
2. hepatitis C infection
3. AIDS
4. pre-existing lymphoma or sarcoidosis
5. graft versus host disease
6. use of anticholinergic drugs

The AECC was used in this thesis. Any cases seen in the SS clinic prior to the AECC acceptance were reevaluated and judged based on the new criteria.

## 2.2 Epidemiology of Sjogren's Syndrome

A recent Center for Disease Control (CDC) review of surveys estimated that Sjogren's syndrome may be as prevalent as rheumatoid arthritis in North American societies.<sup>(32)</sup> The prevalence of any autoimmune disease, however, is difficult to determine. Problems with estimates include the relative rarity of these diseases that makes large population studies challenging. Also, there are wide differences in the prevalence among different subgroups and problems with case definition. Finally, there are those whose disease has burnt out and they are hard to identify.<sup>(33)</sup>

Silman in 1993<sup>(33)</sup> analyzed the prevalence of other autoimmune diseases in a Western population and stated the point prevalence as:

RA-----	8.0 in 1000
Ankylosing spondylitis-----	2.0 in 1000
JRA -----	0.7 in 1000 (<15 years)
SLE -----	0.4 in 1000
Systemic sclerosis-----	0.1 in 1000

The recent CDC survey estimated that more than 21% (46.4 million) of American adults suffer from osteo and/or rheumatoid arthritis. Rheumatoid arthritis was estimated to affect 1.3 million adults ( 0.6%). SS was calculated to affect from 0.4 million to 3.1 million adults (up to 1%).<sup>(32)</sup>

### 2.2.1 Incidence of SS

There are very few studies that estimate the number of new cases of SS per year. An incidence estimate was done in Slovenia for pSS by looking at all patients admitted to

the Department of Rheumatology or referred to the outpatient clinic between 2000 and 2003 because of sicca symptoms. Using the validated European criteria for SS,<sup>(34)</sup> the incidence was 3.9 cases per 100,000.<sup>(35)</sup> Pillemer's estimate of the incidence of SS in Minnesota in 2001 was very similar at 4 in 100,000.<sup>(36)</sup> Alamanos et al. studied a Greek population from 1982 to 2003.<sup>(37)</sup> The incidence was calculated as 5.3 cases per 100,000.

### **2.2.2 Prevalence of SS**

The recent CDC analysis is most interesting in that it demonstrates that the prevalence of RA has lessened and that the prevalence of SS may be higher than previously thought in the US. This review of surveys suggests that up to 3.1 million adult Americans (approximately 1%) suffer from Sjogren's syndrome.<sup>(32)</sup> It is also believed that 9 out of 10 of them are women.<sup>(32, 38)</sup>

The prevalence of SS has been studied over the years and differs with criteria used for diagnosis, with the most recent AECC providing the lesser estimates. This can be seen in the Kabasakal et al. study of 2006 in which both the 1993 European criteria<sup>(29)</sup> and the 2002 AECC<sup>(28)</sup> were used to assess prevalence in a population of women in Turkey. They found 1.56% prevalence with the 1993 criteria and a 0.73% prevalence with the AECC criteria.<sup>(39)</sup> Haugen et al. in 2008 also used 2 sets of criteria in studying a population in Norway. The European criteria of 1993<sup>(29)</sup> and the revised European criteria of 1996<sup>(34)</sup> produced respective prevalence estimates of 0.44%-0.22% in the 40-44 year age group and 3.39%-1.40% in the 71-74 year age group in Norway.<sup>(40)</sup> Zhang et al. determined that the prevalence of SS in Chinese adults was 0.77% by Copenhagen criteria<sup>(24)</sup> and 0.33% by modified San Diego criteria<sup>(26)</sup> in 1987.<sup>(41)</sup>

Different countries and different areas within countries also provide different prevalence numbers. Two studies were conducted in England. Bowman et al. (2004) estimated the prevalence of SS in women in Birmingham, UK, at 0.1% to 0.4%, depending on the assumptions used for diagnosis.<sup>(42)</sup> Thomas et al. found a much higher prevalence of 3%-4% in Manchester, UK, in 1998, using the European Community Criteria.<sup>(29)</sup> Swedish adults were studied by Jacobsson in 1989<sup>(43)</sup> using the Copenhagen criteria<sup>(24)</sup> to establish a 2.7% prevalence. In Slovenia, Tomsic et al. in 1999 estimated the prevalence of SS as 0.6%.<sup>(44)</sup> In a closed rural community in Greece, Dafni et al., in 1997<sup>(45)</sup> estimated the prevalence of SS in women to be 0.6% using the 1990 criteria of Europe,<sup>(46)</sup> while 2.99% were suspected of having the disease.

Specialized hospital populations have also been studied. Sanchez-Guero et al. did a prevalence study in 2005<sup>(47)</sup> in ambulatory patients attending a tertiary care centre of rheumatology and internal medicine. The minimum prevalence was 13.3%. Drosos et al. surveyed an elderly population 1988 and estimated the prevalence of pSS as 6.45%.<sup>(48)</sup>

Risk factors for SS include female sex.<sup>(2)</sup> Age of onset is reported variously at two age peaks: 20-30 years and after menopause to the mid-50s,<sup>(5)</sup> or approximately 45 years.<sup>(6-8)</sup> A risk factor for sSS is the presence of another autoimmune disease.

**Table 2.1: Prevalence of Sjogren’s syndrome**

Study	Criteria	Population	Prevalence	Publ. date
Helmick <sup>(32)</sup>	National surveys	USA	<1%	2008
Kabasakal <sup>(39)</sup>	1993, EEC <sup>(49)</sup>	Turkey, females, 18-75 yrs	1.56%	2006
	2002, AECC <sup>(28)</sup>	Turkey, females, 18-75 yrs	0.72%	

Haugen <sup>(40)</sup>	1993, EEC <sup>(49)</sup>	Norway, 40-44 yrs	0.44%	2008
	1993	Norway, 71-74 yrs	3.39%	
Haugen <sup>(40)</sup>	1996 <sup>(34)</sup> Revised European	Norway, 40-44 yrs	0.22%	2008
	1996	Norway, 71-74 yrs	1.40%	
Bowman <sup>(42)</sup>	2002 <sup>(28)</sup> AECC	Birmingham, UK, women 35-74 yrs	<0.1-0.4%	2004
Drosos <sup>(48)</sup>		Greece, elderly, 67-95 yrs	6.45%	1988
Tomsic <sup>(44)</sup>		Slovenia	0.6%	1999
Thomas <sup>(50)</sup>	1993, EEC <sup>(29)</sup>	Manchester, UK	3-4%	1998
Sanchez-Guero <sup>(47)</sup>	2002 <sup>(28)</sup> AECC	Ambulatory rheum patients	13.3%	2005
Dafni <sup>(45)</sup>	1990, EEC <sup>(49)</sup>	Greek women, 18-90 yrs	0.6%	1997
Jacobsson <sup>(43)</sup>	1986 <sup>(24)</sup> Copenhagen	Swedish adults, 52-72 yrs	2.7%	1989
Zhang <sup>(41)</sup>	1986 <sup>(24)</sup> Copenhagen	China, adults	0.77%	1995
	1986 <sup>(26)</sup> mod San Diego	China, adults	0.33%	

Although the prevalence of SS is difficult to know, it appears that from 0.1% to 4% of the populations studied suffer from this disease. The diagnosis remains elusive in many circumstances because of the relatively benign presentation.



### **2.2.3 Epidemiology of secondary Sjogren's syndrome**

The prevalence of secondary SS (sSS) has also been studied in various rheumatic populations. Coll et al. 1987<sup>(51)</sup> took 78 autoimmune (AI) patients (RA, scleroderma, sicca syndrome, PBC, other AI) and 33 non-AI patients and found the prevalence of sSS to be 62% in RA and 69% in scleroderma. The criteria used were low Schirmer scores and rose bengal staining, and labial gland biopsy and scintigraphy of salivary glands.

Secondary SS in RA was studied in 1988 in RA patients in Greece by Andonopoulos et al.<sup>(52)</sup> They found a prevalence of 31% in RA.

Drosos studied sSS in progressive systemic sclerosis in 1988.<sup>(53)</sup> Their estimate was 20.5% when studying a small group of 44 sequential patients.

Andonopoulos in 1990 studied the prevalence of sSS in systemic lupus erythematosus (SLE) in Greece.<sup>(54)</sup> Their estimate was 8.3% prevalence.

Thus the prevalence of sSS in autoimmune disease has been shown to vary from 8% to 69% and is perhaps overlooked because of the overriding systemic disease characteristics of the primary disease.

## **2.3 Etiology of Sjogren's Syndrome**

As with other autoimmune diseases, how Sjogren's syndrome begins is not known. It is believed that multiple factors including inflammation, viral invasion, hormonal imbalance and genetics may be involved in the process that tips the balance of immunity in the salivary and lacrimal glands.

It is this author's clinical experience that SS patients can describe an acute onset of their disease. They speak of a severe flu-like episode that was followed by dryness and the diagnosis of SS. The majority of patients, however, suggest that they had ongoing

symptoms of dryness and a general sense of unwellness for some years before their diagnosis. Perhaps these two presentations represent examples of the two most likely etiologies of SS: acute viral invasion of the exocrine glands versus the slower onset of prolonged mild inflammation that leads to a tipping point of autoimmune disease.

Although SS is a systemic disease, its origins are the lacrimal and salivary glands. In keeping with the emphasis of this thesis on ocular pathophysiology, the following etiological discussion will deal mainly with the inflammation of the lacrimal gland. It is clear that the salivary gland has been more rigorously studied because of the availability of minor salivary glands for biopsy. However, it is assumed that the inflammation of the lacrimal gland parallels that of the salivary glands, despite their obvious differences in form and function.

Drastic changes occur in immune homeostasis in the lacrimal glands before SS patients present in our offices with symptoms of dry eyes and blurred vision. Both T and B lymphocytes are found in larger numbers and are highly active, and the secretory acinar cells have changed in form and function. As mentioned above, there are two major theories as to etiology: viral infection of the glands and a failure of local immune regulation. The influence of hormones, the thymus and cytokines will also be discussed.

### **2.3.1 Viral theory**

Viral invasion of the lacrimal gland could certainly cause an autoimmune response. Both T and B immune cells are present in the healthy lacrimal gland and react when viruses invade. Early viral theories concentrated on the cytomegalovirus (CMV).<sup>(55)</sup> Current theory, however, has shifted focus to Epstein-Barr virus (EBV). Once EBV invades lacrimal acinar cells, physiology is altered as viral mechanisms function to gain

control of all cellular resources. Once this process occurs and acinar cells begin to produce and display viral proteins, local lymphocytes initiate a response involving the production of inflammatory chemicals that will help to destroy both the virus and the host cell. The displayed viral proteins can mimic acinar self-proteins, and thus the inflammation induced by a virus can transform into an autoimmune process.<sup>(56, 57)</sup> Viral invasion of these lacrimal cells also promotes the cellular production of autocrine growth factors that increase the number of active lymphocytes within the gland.<sup>(58, 59)</sup> These viral-induced autocrine growth factors are associated with the specific type of B cell activity seen in SS that accounts for a 40-fold greater chance of B cell malignancies occurring in SS patients.<sup>(60)</sup>

The potential relevance of EBV as an initiating factor in SS is highlighted by two studies that have characterized the appearance of SS following infectious mononucleosis.<sup>(61, 62)</sup> Additional work has localized EBV virus in the glands and blood of SS patients.<sup>(63)</sup> Fox et al.<sup>(64)</sup> and Mariette et al. detected EBV in salivary glands of SS patients.<sup>(65)</sup> Pflugfelder detected EBV genes in 8 of 10 lacrimal glands from Sjogren's syndrome patients who were seropositive for EBV antigens.<sup>(66)</sup> They also found EBV antigens in 2 of 6 Sjogren's lacrimal glands and in none of the controls and noted a correlation between levels of serum antibodies to EBV antigens and the severity of aqueous deficiency.<sup>(67)</sup>

Viral inflammation cannot, however, account for all cases of SS as evidence of EBV is not present in all SS lacrimal and salivary glands. In addition, EBV genomic sequences are frequently seen in normal lacrimal tissue. In fact, Deacon et al. found that EBV DNA can be detected with equal frequencies in normal as well as primary and

secondary SS tissue.<sup>(68)</sup> This has led to the suggestion that it is possible that reactivation of EBV is a consequence rather than a cause of immunoproliferation in the salivary and lacrimal glands in SS.<sup>(69)</sup>

Retroviruses have also been discussed as initiators of SS. Research on AIDS and HIV-positive patients has contributed to this discussion. The number of HIV patients with dry eye disease confirmed with Schirmer testing is as high as 21%.<sup>(70)</sup> Garry et al. noted particles antigenically similar to the HIV virus in extracts of salivary glands in 2 of 6 SS patients.<sup>(71)</sup> AIDS patients with xerostomia did show lymphocytic focus scores of 2 or more in the salivary biopsies, but the cells were different from those in SS in that the predominant T cell was of the suppressor subset in contrast to the helper subset described in SS.<sup>(70)</sup> Thus, the altered immune system in AIDS gives a similar pattern of inflammation within the salivary gland, but the foci are composed of different cells, highlighting the uniqueness of SS inflammation.

### **2.3.2 Acinar cell theory**

There is, however, growing evidence that autoimmunity may occur at a local level without viral invasion. Sjogren's syndrome, then, becomes a disease of failed local immunity. In this theory acinar cells themselves become antigen presenting cells. Bottazzo and co-workers,<sup>(72)</sup> when studying thyroid disease, first put forward the theory that epithelial cells that express major histocompatibility complex (MHC) class II molecules promote autoimmune inflammatory activity. MHC class II positive epithelial cells initiate an autoimmune response by presenting their own internal proteins on their surfaces to reactive T lymphocytes and thus activate these cells at the local level. This

form of inflammation has been noted in several ocular conditions including corneal transplant rejection,<sup>(73)</sup> uveitis<sup>(74)</sup> and proliferative vitreoretinopathy.<sup>(75)</sup>

Lacrimal secretory cells, by nature, have an immunologically precarious relationship with their surrounding tissues that is a function of their normal secretory activities.<sup>(76)</sup> Acinar cells have ongoing communication between their apical and basal membranes and with the interstitium, as a function of packaging and secreting secretory IgA (sIgA) and prolactin.<sup>(60, 77)</sup> MHCII molecules that are made in small amounts by the healthy acinar cell can travel with sIgA or prolactin and can attach to autoantigens on their way.<sup>(78)</sup> The MHCII presentation of autoantigens is therefore a fairly common occurrence in the lacrimal gland, as there is a basal level presentation that remains under control because the mechanisms to prevent a tip-over into autoimmunity are in place.

Prevention of auto-immune inflammation involves the dendritic cells. The dendritic cells in the lacrimal gland could engulf these self-proteins, take them to the local lymph nodes and direct T cells to return to the gland to cause an immune inflammation. However, in the normal state of affairs the local dendritic cells mature under the influence of local, calming autocrine factors, expressed by the acinar cells. They therefore circulate through the local lymph nodes and direct T cells to become regulatory T cells as opposed to inflammatory helper T cells. Hence no immune inflammation occurs.

There is a clear breakdown in this protective immune system when SS presents. Mircheff and others<sup>(78)</sup> have proposed a theory to explain the lacrimal gland instigator factor. Their theory suggests that under duress, lacrimal gland epithelial cells begin to mimic dendritic antigen-presenting cells through an excess presentation of self-antigens.

Thus at a local level the acinar cells stimulate naïve T cells within the gland to initiate an autoimmune inflammatory cascade by duplicating themselves and stimulating B cells. The change in function of the acinar cells is likely a result of prolonged low-grade inflammation that creates a cytokine environment that encourages the acinar cells to behave like antigen presenting cells (APC). This theory is based, in part, on the observation that salivary gland epithelial cells of most SS patients display MHC class II molecules.<sup>(69, 79, 80)</sup> Although the same uniformity is not found in all lacrimal gland cells of SS patients,<sup>(81)</sup> the theory may still apply in many cases of SS.

### **2.3.3 Thymus theory**

Although local immune theories of SS are intriguing, some theorists believe that a more central and fundamental deficiency exists in the immune system in SS. This theory looks at the thymus for answers. The first step in the failure of any self-tolerance could begin with a failure of the thymus to eliminate the T cells that have potential self-reactivity.<sup>(82-84)</sup> If we assume that the epithelial cells of the lacrimal gland have displayed self-protein and that these antigens have been delivered by antigen presenting dendritic cells to the T cells in the thymus, why does the thymus not eliminate these destructive cells?

One of the major functions of the thymus is the elimination of such auto-stimulated T cells through “negative selection.”<sup>(84)</sup> Numerous signals such as fas, fas-ligand, bax and bcl-2 determine whether the potentially self-destructive T cells are released into the periphery or undergo apoptosis.<sup>(85)</sup> This selection process appears disturbed in autoimmune diseases. For example, in mice, bcl-2 is over-expressed as

autoimmune disease severity increases. The imbalance or over-expression of bcl-2 may then allow the escape of those T cells that have been activated by lacrimal self-proteins.

### **2.3.4 Hormonal influence**

Hormones appear to be another factor in the march towards autoimmune inflammation. Since SS<sup>(86)</sup> and other forms of dry eye disease are more common in women, hormonal imbalance has been implicated in this process.<sup>(87, 88)</sup> A healthy lacrimal gland requires a balance of hormonal stimuli to maintain its form and function. The effect of hormonal imbalance on dry eye disease seems obvious at a clinical level. Female patients present with dry eye symptoms in certain hormonal states including lactation, pregnancy, post-menopause, oral contraception use, supplemental estrogen use<sup>(89-91)</sup> and premature ovarian failure.<sup>(92, 93)</sup> One proposed commonality of these altered hormonal states is low testosterone bioavailability.

Indeed, androgens appear to have the greatest influence on the health of the lacrimal gland,<sup>(94)</sup> and a large body of literature on this subject has been reviewed by Sullivan.<sup>(95)</sup> In humans, both androgen receptor mRNA and androgen-binding sites have been localized in the lacrimal gland.<sup>(96)</sup> Androgen stimulation of acinar cells via specific receptor activation has a significant influence on protein synthesis in these cells.<sup>(97)</sup> As such, androgen deficiency may result in the inability of the acinar cells to produce and secrete their contents. Androgen levels are decreased in both SS and systemic lupus erythematosus (SLE).<sup>(98, 99)</sup>

The effect of estrogen on the lacrimal gland is far more controversial. At a clinical level, the results of epidemiological studies suggest that women on estrogen replacement therapy have more symptoms of dryness than various control groups.<sup>(100)</sup> However,

estrogen influence on the lacrimal gland has been difficult to characterize experimentally. Neither Warren<sup>(101)</sup> nor Cornell-Bell et al.<sup>(102)</sup> were able to demonstrate estrogen receptor protein in the human lacrimal gland despite the positive identification of receptor mRNA.<sup>(103, 104)</sup> It may be that estrogens influence the lacrimal gland only indirectly by suppressing ovarian androgen production and increasing pituitary prolactin production.<sup>(105, 106)</sup>

Prolactin is also an important hormone in lacrimal gland function. It is both synthesized and secreted by the lacrimal gland, and these glands are positive for prolactin receptors.<sup>(107, 108)</sup> Prolactin is known to influence a good number of autocrine and paracrine functions in a wide variety of tissues.<sup>(109)</sup> Of particular interest is prolactin's role in the mammary gland, where it influences the homing of IgA secreting B and plasma cells that infiltrate that gland.<sup>(110)</sup> In the immune system prolactin can stimulate T and B cell proliferation and influence the maturation of T cells.<sup>(111)</sup>

Prolactin has been shown to influence tear function at a clinical level in that increased levels of prolactin in women were found to be associated with several measures of reduced tear fluid production.<sup>(112)</sup> Rabbit studies have shown that increased levels of prolactin, which occur in pregnancy and lactation, cause the lacrimal gland to undergo both immunoarchitectural changes<sup>(89)</sup> and functional changes.<sup>(113)</sup> Recent work with rabbits has shown that over-expression of prolactin reorients the position of secretory vesicles from their normal place at the apical membrane of the acinar cell to the basal-lateral membrane.<sup>(109)</sup> Prolactin also influences secretion through its effect on cholinergic stimulation of the gland. In fact, it appears that cholinergic receptor expression may be dependent on prolactin.<sup>(114)</sup>



There is a critical level of hormonal support required for the lacrimal gland to remain healthy and free of autoimmune disease. Androgen support and a normal level of prolactin appear to be the most critical hormones for lacrimal gland function.

### **2.3.5 Genetic influence**

A genetic predisposition to SS has been suggested on the basis of familial aggregation, candidate genes and animal model studies.

The inflammatory rheumatic disorders, which include SS, have similar clinical presentations and autoantibody profiles. Systemic lupus erythematosus is considered the prototypic disease of this type and it often shares many SS characteristics.<sup>(115, 116)</sup>

To understand the genetic influence on SS, familial studies have been undertaken and several families having 2 or more cases have been described.<sup>(117-119)</sup> Twins exhibited a very similar phenotype with clinical presentation, immunoglobulin profiles and salivary gland focus scores showing near identical results.<sup>(120, 121)</sup>

Familial clusterings of different autoimmune diseases have been reported. It has been noted that 30%-35% of SS patients have relatives with other autoimmune diseases.<sup>(117, 122, 123)</sup> SS itself can be found in its secondary form in the same body, associated with other autoimmune diseases.<sup>(28)</sup>

Genetic studies have suggested that there may be a cluster of major Histocompatibility complex (MHC) genes that can influence the propensity for autoimmune disease. It is suggested that, in some cases, clinically distinct autoimmune diseases may be controlled by a common set of these susceptibility genes.<sup>(115, 124-127)</sup>

The role of MHC proteins in the immune system was first recognized by Benacerraf in 1981.<sup>(128)</sup> These MHC proteins are expressed by the cells of all higher

vertebrates, and in humans they are called human leukocyte-associated antigens (HLA). The genes that encode the MHC proteins are some of the most polymorphic in the human genome. There are two classes of MHC proteins, and the most common reports in autoimmune diseases are those specific to MHC class II molecules. It is believed that specific MHC II alleles determine the targeting of specific autoantigens, resulting in disease-specific associations.<sup>(125)</sup>

The class II genes are the ones most linked to SS as well, specifically the HLA-DR and HLA-DQ alleles.<sup>(129, 130)</sup> Distinct HLA haplotypes have been associated with certain autoantibodies in SS.<sup>(130-134)</sup> It is believed that these genes confer risk<sup>(135)</sup> as well as severity of the disease in some groups.<sup>(136)</sup>

Thus humans with a particular form of MHC II molecules are more likely to pick up ribonuclear associated proteins such as Ro and La because of their configuration and thus more likely to take these proteins to the surface of the lacrimal cells and cause SS autoimmune inflammation.

Since cytokines mediate much of the disease process in SS, studies have been undertaken to determine the genetics of the expression of these proteins. There is some suggestion that genes for TNF- $\alpha$  may play a role in SLE.<sup>(137)</sup> However, to date no convincing relationship with SS and cytokine polymorphism has been discovered.<sup>(138)</sup>

Apoptosis irregularities have been suggested to be a part of the SS pathophysiology,<sup>(139)</sup> and therefore studies have undertaken to look at apoptotic genes. Mutations in these genes have been implicated in human disease.<sup>(140)</sup> Of particular interest are the Fas genes, and mutation in this gene has been identified as a contributor to autoimmune lymphoproliferative syndrome.<sup>(140)</sup> Polymorphisms in Fas and FasL genes

have been identified in primary SS.<sup>(141)</sup> However, no correlates with these genes and differences in proteins have been identified.

Nakamura et al. found that alleles on the X chromosome over-expressed anti-apoptotic proteins in SS, thus adding to the understanding of the high prevalence of this disease in women.<sup>(142)</sup>

Genes that encode the transporter molecules that move antigens within the cytoplasm have also been studied. The question of whether a defect in these molecules might cause antigens such as Ro and La to be more easily moved to the cell surface has been explored. The transporter molecule genes have been suggested as possibly being associated with SS susceptibility.<sup>(143)</sup>

Kaschner et al.<sup>(144)</sup> looked at immunoglobulin genes and determined that there was a disturbance of B cell maturation and abnormal editing of immunoglobulin receptors that may contribute to SS.

Mice have demonstrated that chromosomes 1, 4 and 10 hold major susceptibility loci for autoimmune saladenitis.<sup>(145-147)</sup>

Generally there is a belief that certain people are more susceptible to autoimmune disease because of their genetic makeup. Much work is still to be done in this area.

## **2.4 Pathophysiology of the Lacrimal Gland**

Of the many dry eye patients who present in our offices, a cohort will have Sjogren's syndrome. The Schirmer test will attest to the fact that their lacrimal glands are not secreting properly. This is a result of a specific type of lymphocytic infiltration that occurs following a breakdown in the normal immunohomeostatic state of this active secretory gland. Genetic and hormonal influences are likely a part of this road to

autoimmunity. Whether through a viral invasion or an accumulation of excess stimuli, the acinar cells enhance their cytokine expression and present self-proteins on their surfaces. These events lead to lymphocytic trafficking to the inflamed area. From this beginning comes the active destruction of both form and function of the lacrimal gland. However, the gland remains a main participant in the autoimmune process as the B lymphocytes that are activated and created within the gland affect other parts of the body, including the salivary and parotid glands as well as the ocular surface, where both the absence of complete tears and the activity of lymphocytes and their cytokines cause malfunction of the epithelium and its secretory components.

The inflammatory activity that results in patients presenting with ocular surface disease and low Schirmer scores in SS is complex and not fully understood. It is believed that the disease begins with lacrimal gland inflammation. Animal studies and a small number of histological observations of human lacrimal glands give us a good deal of insight into the type of inflammation that occurs in these glands in SS. However, the most common and detailed description of SS inflammation comes from the study of human salivary glands, and much of the discussion of the pathophysiology of SS derives from this body of literature. It is believed that similar events are occurring in the lacrimal gland, but it is clear that these glands are not identical and therefore not all of the salivary gland results will translate to the lacrimal gland.

It is believed that the ocular system evolved because vision allows survival of some species, including the human species. The survival activities of hunting and gathering food, avoiding enemy attacks and socializing can be considered some of the most important functions of this system. To perform these tasks, the visual system must

maintain a clear cornea. The challenge of these tissues is that they are continuously exposed to the environment of toxins and microbes. The tear film is an essential element in the protection of the cornea and the lacrimal gland is one of the most important sources of tears. This process of immune regulation of the ocular surface can dysfunction and create autoimmune inflammation.

The lacrimal gland is a part of the mucosal ocular lacrimal system that includes the tears, conjunctiva and cornea and lacrimal duct.<sup>(148)</sup> Because of the exposure of this system to both external and internal environments, it maintains a complicated immunoregulatory system.<sup>(148-153)</sup> The most common entry points in our bodies for antigens are the mouth and gut mucosal systems. It is believed that antigens received by these systems cause the generation of Th2-type CD4+ T cells and IgA producing B cells to be formed within the lymph system that then travel to distant IgA effector tissues such as the salivary and lacrimal glands, where the B cells secrete their protective IgA onto exposed surfaces such as the ocular surface.<sup>(154)</sup> The Th2 cells in these glands continue to encourage B cells to become sIgA secreting plasma cells, which are an essential part of the protection of the ocular surface.<sup>(155)</sup>

This type of immune physiology occurs in the salivary,<sup>(156)</sup> respiratory, gastrointestinal and urogenital tracts of the body as well.<sup>(157)</sup> This is one of the most important barrier functions of the mucosal immune system: to maintain a barrier against external pathogens while avoiding a major inflammatory event that would impair normal parenchymal function.<sup>(157)</sup>

In SS there is a breakdown of this barrier function, and the resulting infiltration of the secretory lacrimal and salivary glands that occurs is active, chronic and progressive.

The inflammation involves several cell types, and there is some argument as to whether SS should be designated a disease of T cells, B cells or epithelial cells. The importance of dendritic cells and vascular endothelial cells must also be included in the discussion of this complicated autoimmune inflammation. Finally the role of cytokines, chemokines and hormones cannot be overlooked.

### **2.4.1 Cells**

The healthy lacrimal gland contains a small number of T lymphocytes and an even smaller number of B lymphocytes.<sup>(158)</sup> The T lymphocytes are represented by regulatory T cells (Treg),<sup>(159)</sup> which outnumber the helper or effector cells (Th) by a 2- to 3-fold margin,<sup>(160)</sup> keeping the lacrimal gland free of any gross inflammatory response. The number and the balance of lymphocytes within the gland is a key to its health and function. These immune cells are present to control any foreign invasions, particularly those triggered by microbes. However, they must not upset the balance of inflammation to the point of lacrimal gland destruction. This immune homeostasis requires a tension between two conflicting imperatives: responding to foreign antigens and avoiding destructive reactions to autoantigens.<sup>(161)</sup>

### **2.4.2 T cells**

Young T cells travel from the thymus to the lymph nodes, where they meet up with the dendritic cells that have come from the lacrimal and salivary glands. How the T cells differentiate at this point is a function of which antigens the dendritic cells present and whether the dendritic cells have matured to be peace-loving, in which case they will promote the maturation of Tregs, or more inflammation-loving, which will promote the maturation of Th1 or Th2 cells.

When regulatory T cells are stimulated by antigens, they secrete IL-10 and TGF- $\beta$ .<sup>(162)</sup> These are calming cytokines that have been shown to repress lymphocytic proliferation in vitro.<sup>(163)</sup> In the lacrimal gland their presence allows the dendritic cells to mature with tolerogenic phenotypes and influence the interaction between memory T cells and mature dendritic cells that will prevent the memory T cells from being activated to Th cells and perhaps allowing them to express regulatory cytokines. As Mircheff has stated: “The cycles of successive generations of dendritic cells and regulatory T lymphocytes appear to constitute a positive feedback loop that enforces tolerance to the lacrimal autoantigens.”<sup>(157)</sup>

In SS salivary and lacrimal gland lesions, the effector T cells, particularly the CD4+Th2 cells, are found in very high numbers and dominate the lymphocytic infiltrate.<sup>(81, 164, 165)</sup> They are believed to be a major player in the induction phase of the disease<sup>(166)</sup> and are present as the infiltrate progresses.<sup>(167)</sup> The influence of these activated effector T cells is to secrete cytokines that allow the inflammation to progress and to stimulate B cells to secrete immunoglobins to self-proteins like Ro and La.

To add to the intensity of this inflammation in SS, there is a dearth of regulatory T cells in the salivary and lacrimal glands.<sup>(168)</sup> The cause of the reduced number of Treg cells is not known, although there is some suggestion that low levels of expression of TGF- $\beta$  may be a factor.<sup>(169)</sup> TGF- $\beta$  is strongly expressed in ductal epithelial cells of healthy salivary glands but not in primary SS.<sup>(169-171)</sup> The lack of Treg cells in the salivary glands contributes to the increased expression of anti-Ro and anti-La by B cells that now lack the calming control of Treg cytokine expression<sup>(172, 173)</sup> and are subject to the stimulatory effects of the Th2 CD4+ cells.

### 2.4.3 B cells

B cells migrate from bone marrow to organized mucosa-associated lymphoid tissue (MALT) and finally to end organs such as the lacrimal gland. The healthy lacrimal gland maintains calm control of itself, in part, by creating a molecular environment that directs B cells to mature to plasma cells that express secretory IgA, the protective immunoglobulin that bathes the ocular surface. The molecules responsible for this preferred maturation process are TGF- $\beta$ , IL-6 and prolactin. The expression of dimeric IgA (dIgA), which in turn provides secretory IgA, by the lacrimal gland B cells creates an anti-inflammatory environment as well because this form of IgA is anti-inflammatory in itself,<sup>(174, 175)</sup> as it does not fix complement and thus avoids humoral inflammation.<sup>(176-178)</sup>

Younger, naive or memory B cells are also present in the lacrimal gland in case they are needed to secrete immunoglobins against invading pathogens. This stimulus to secrete comes from the foreign antigen itself and T helper cells. The B cells so stimulated become effector cells and finally plasma cells.<sup>(179)</sup>

In SS, the infiltrates in the secretory glands are composed, in part, of B cells, some of which are believed to come from peripheral sources while others are created within the gland.<sup>(180-182)</sup> These B cells secrete IgG and IgM.<sup>(180, 181)</sup> The B cell proliferation and recruitment in SS salivary and lacrimal glands seems directed and specific in that peripheral serum B cells of SS patients show reduced numbers of B memory cells while there is a marked accumulation of these cells in the salivary gland.<sup>(183)</sup> The sIgA secreting B cells remain in the gland, but instead of being dominant are now dominated by the number of active Ro and La immunoglobulin secreting B cells. They are generally found in the tissue peripheral to the active infiltrate.<sup>(184)</sup>



The activity of B cells in SS is well studied. They show abnormalities in the peripheral blood and in the local environment of the salivary glands.<sup>(184, 185)</sup> These B cells undergo clonal expansion<sup>(186)</sup> and increased plasma cell occurrence.<sup>(187)</sup> The serum of SS patients shows a resulting increase in levels of gammaglobulin. This clonal proliferation and hypermutation of B cells in the salivary glands of SS patients is well documented.<sup>(188, 189)</sup> There is also direct and indirect evidence of local production of autoantibodies.<sup>(182, 190, 191)</sup> These SS B cells are particularly prone to expressing immunoglobins to Ro and La.<sup>(184)</sup> These two ribonucleoproteins are found in the nucleus and cytoplasm of all cells.<sup>(192, 193)</sup> The Ro and La immunoglobins that are present in the serum are believed to be responsible for many of the systemic manifestations of SS.<sup>(194)</sup>

Several other antibodies are secreted by the B cells in SS. These include antibodies to  $\alpha$ -fodrin<sup>(195, 196)</sup>, actin,<sup>(197)</sup> telomere<sup>(198)</sup> and M3 muscarinic receptors.<sup>(199)</sup> The M3 autoantibodies are believed to play a large role in the neurological inhibition to secretion in the lacrimal and salivary glands of SS patients.

#### **2.4.4 Dendritic cells**

Immature dendritic cells cycle from bone marrow through the lymph system to effector sites where they sample foreign antigens and autoantigens.<sup>(179)</sup> Then they mature and emigrate to the lymph nodes, where they influence the maturation and differentiation of T cells.

Dendritic cells are considered by some to be the “sentinels” of the immune system.<sup>(200)</sup> Although dendritic cells can easily capture apoptotic cell proteins and present self-antigens, they tend to exert mainly tolerogenic functions in physiological situations.<sup>(201)</sup> Thus they have the capacity to promote peripheral self-tolerance by

helping naïve effector T cells to differentiate to Tregs that will then secrete IL-10 and keep the gland calm.<sup>(201-203)</sup> This calm environment is also promoted by lacrimal gland epithelial cells themselves that express TGF- $\beta$  and prolactin.<sup>(107, 204)</sup> There is also some evidence that they express IL-10.<sup>(157)</sup> All of these expressions promote the maturation of modulating dendritic cells.

The normally functioning lacrimal gland creates situations where dendritic cells are exposed to local autoantigens that have made their way into the interstitium during IgA transcytosis. Despite this risky reality, autoimmune disease does not occur in most individuals because there is this calming mechanism in place that allows for a “normal” level of autoantigen exposure to be experienced without tipping the immune balance over into an acute autoimmune inflammation. Thus, despite this common exposure of self-antigens, the healthy lacrimal gland avoids inflammation because acinar cells maintain a local signaling milieu that allows dendritic cells to mature with tolerogenic properties even after they have taken up autoantigen.<sup>(201)</sup>

This protective mechanism has been studied in other tissues as follows. High local concentrations of IL-10 in the upper respiratory tract lead to the generation of regulatory T cells.<sup>(205)</sup> Also, TGF- $\beta$  has been shown to influence dendritic cells to promote regulatory T cells in the synovium.<sup>(206, 207)</sup>

When dendritic cells lose their tolerogenic functions, they participate in inflammation. Microbial antigens cause them to mature and react rapidly.<sup>(208)</sup> At this maturation point they begin to secrete IL-1, IL-6, IL-12, IL-18 and IL-23, which are pro-inflammatory. They then move into adjacent lymph nodes and scan for naïve T cells and induce them to become effector cells to that antigen.<sup>(209)</sup> These pro-inflammatory changes

in the glandular cytokine environment influence dendritic cells to change their ways at a local level and present more self-antigens to T cells and influence their transition to effector cells.<sup>(210)</sup>

The dendritic cells of the ocular surface may contribute to the inflammation of the lacrimal gland as well.<sup>(211)</sup> The cornea and conjunctiva are exposed to many toxins in their environment and can become inflamed. The subsequent secretion of inflammatory cytokines on the ocular surface can cause conjunctival dendritic cells to load up on autoantigens and become aggressive cells that are refractory to the regulatory T cells in the area. They would then go to the lymph nodes and spleen to generate T cells that are autoantigen-specific effector T cells. Mircheff<sup>(157)</sup> believes that the accessory lacrimal glands may become a part of this process by exposing their cryptic epitopes, such as Ro and La, to dendritic cells that could then educate T cells in the lymph nodes to recognize these once hidden proteins and go to the lacrimal gland proper.

#### **2.4.5 Acinar cells**

Acinar cells contribute to SS inflammation in two ways. As mentioned above, they may allow dendritic cells to be exposed to self-antigens through the normal delivery of IgA<sup>(212, 213)</sup> and the spill-over that may occur of internal cell proteins. These acinar cells have ongoing communication between their apical and basal membranes and with the interstitium.<sup>(77), (60)</sup> MHCII molecules can travel with these molecules and can attach to autoantigens on their way.<sup>(78)</sup> This ongoing exposure of autoantigens within the lacrimal gland remains contained and controlled in the healthy lacrimal system. The exposure of autoantigens occurs when a vesicle fuses with the basal-lateral membrane to insert empty IgR. At this moment there is a spill-out of fluid that contains soluble

autoantigens that have been carried from the endosomes where vesicles form. The autoantigens get there via many trafficking events that are a normal part of the acinar cells' secretory work. This is true of all secretory cells; however, the lacrimal gland may be particularly prone to excess exposure because it has to secrete so much IgA and therefore more autoantigens.<sup>(214-216)</sup>

The other mechanism that can create autoantigen exposure is overstimulation of the lacrimal gland. For example, if the ocular surface became chronically inflamed through environmental stimuli, for example, the nociceptors would be chronically stimulated. The lacrimal acinar cells that respond to such stimuli are at particular risk of excess autoantigen exposure under these circumstances because of the dynamic nature of traffic into the lysosomal pathway. When asked to secrete, the cells redirect traffic from the lysosome to transytotic, merocrine and recruitable secretory vesicles.<sup>(217-220)</sup> This keeps up to such a degree that many proteins in the lysosome reflux to the TGN, to immature vesicles and to endosomes. This allows the self-proteins that were once so carefully destroyed and protected to make their way to the cell surface.<sup>(221)</sup>

Thus as this inflammatory process continues, the acinar cells themselves become an active source of excess antigen presentation.<sup>(60)</sup> Certainly, the salivary gland epithelial cells of most SS patients have been shown to display MHC class II molecules.<sup>(222-224)</sup> Although the lacrimal gland is not specialized in the presentation of MHC class II molecules, it is believed that in SS the cytokine environment changes because of a local inflammatory event, causing the expression of interferon gamma (IFN- $\gamma$ ) by local inflammatory cells. INF- $\gamma$  has been shown to encourage epithelial cells to express class II

molecules in much higher concentrations.<sup>(72, 225)</sup> The INF- $\gamma$  can be expressed locally or may make its way from an inflamed salivary gland.

The increased expression of MHC class II molecules alone is not enough to create a SS autoimmune response within the gland as these carriers must also connect to self-proteins like Ro and La and take them to the membrane of the cell. Our cells are protected in many ways from allowing the class II molecules to take self-proteins to the surface. However, stressful physiological situations may change the orderly building and breakdown of proteins within the cells. This is an exciting concept from a clinical point of view. That is, the lacrimal gland itself, under stress to over-perform, might lend itself to autoimmune inflammation. For example, during stressful life situations and excess crying, or in low hormonal states, during which time the gland cannot function well, thus inducing dry eye symptoms, there may be secretory stimulating neurons firing at elevated levels for sustained periods of time. This stressful experience of acinar cells attempting to keep up with the demand of secretion could produce an unusually large amount of newly synthesized proteins that accumulate within the cell. The chaos of too much protein would lead to improper trafficking within the cell compartments, the attachment of class II molecules to self-proteins and the subsequent appearance of these proteins on the cell surface.

#### **2.4.6 Vascular endothelial cells: HEVs**

Changes in vascular endothelial cells occur in SS that allow the accumulation of lymphocytes within the salivary and lacrimal glands. Lymphocytes with potential autoimmune reactivity that escape the thymus and enter the circulation must bind to very specific high endothelial venules to avoid destruction.<sup>(226)</sup> The cytokine profile of the

lacrimal gland in SS allows such high endothelial venules (HEVs) to form.<sup>(227)</sup> These venules over-express adhesive molecules, including integrins, V-CAMs, selectins and cadherins, that promote the entry of the autoinflammatory lymphocytes into the gland and also help to protect them from apoptosis.<sup>(227)</sup>

#### **2.4.7 Germinal formation in SS**

The myriad factors that are involved in this autoimmune inflammation could suggest that there is a randomness at work here. However, histological studies of the salivary glands in SS suggest that there is a meaningful architecture to this inflammation once it gets going. This architecture is fully formed when germinal centres (GC) are created. These centres are actually mini-lymph nodes that maintain the inflammatory process in a self-sustaining fashion.

The presence of lymphoid follicles has been observed in other autoimmune diseases such as the thyroid in Hashimoto's disease,<sup>(228)</sup> the thymus in myasthenia gravis<sup>(229)</sup> and the synovium in rheumatoid arthritis.<sup>(230-232)</sup> In all of these cases, it is believed that local infection, inflammation or cell destruction can lead to an excess exposure of previously ignored self-antigens that directly triggers lymphoid neogenesis and autoimmune reactions in the tissues where self-antigen is continuously exposed.<sup>(233)</sup>

The road to germinal centre formation is described as follows: "During the immune response against self-antigens, B cells bearing the antigen-specific receptors are stimulated to proliferate and differentiate into Ab-secreting plasma cells within the germinal centers.<sup>(234)</sup> This requires the presence of follicular dendritic cells (FDC) and activated CD4+ Th cells, CD40/CD40 ligand interaction, and a cocktail of cytokines to create an environment where germinal center reaction can take place."<sup>(234)</sup>

Thus early in SS inflammation of the lacrimal gland, a few B cells bearing Ro and La antigen receptors are stimulated to undergo clonal proliferation in the lymphocyte foci and to differentiate into effector cells (centroblasts, centrocytes), memory B cells and plasma cells. The mature plasma cells then migrate out of the area into the surrounding tissue. The B cells that express the Ag receptors for Ro and La are selected by competition for Ag on the surface of local dendritic cells and are rescued from apoptosis.<sup>(235)</sup> Thus there is a stimulus to mature as self-antigen plasma cells, a stimulus to proliferate and a protective environment for survival.

The salivary gland has been the source of investigation of germinal centres in SS.<sup>(188, 234, 236-239)</sup> Salomonsson et al. determined that 17% of the SS minor salivary gland biopsy samples have GCs.<sup>(240)</sup> These GCs were found to be composed of aggregates of T and B cells with a network of follicular dendritic cells and activated vascular endothelial cells. These centres showed functional characteristics of production of anti-Ro and anti-La and mild apoptotic events (TUNEL stain). Also those patients whose biopsies demonstrated GCs had a much higher concentration of anti-Ro and anti-La in the serum.<sup>(240)</sup>

The formation of GCs is complex, orchestrated by chemokines, cytokines and adhesion molecules.<sup>(241)</sup> These GCs have similar features to secondary lymphoid organs that include activated post-capillary high endothelial venules, GCs in the interface of B and T cell areas, expression of adhesion molecules and chemokines mediating the homing of naïve cells.<sup>(242, 243)</sup>

Salomonsson found that those patients with GC formation had them in more than 1 place.<sup>(240)</sup> A mass of cells is required to provide the microenvironment for GC

formation, and when it is initiated, the GC formation is a general phenomenon occurring at multiple sites. B cells producing Ro and La were more frequent in patients with GCs, so it appears that the GCs have functional activity. These GCs may also provide an environment for the survival of long-lived plasma cells, as has been suggested by other inflamed tissue.<sup>(244)</sup>

The molecular environment of cytokines and chemokines is critical to “tertiary” lymphoid organogenesis in the exocrine glands in SS.<sup>(245, 246)</sup> Chemokines, in particular the chemokines CXCL13, CXCR5 and CCL21, have been shown to be expressed in the glandular infiltrate of SS and are believed to participate in maintaining the ongoing local antigen driven polyclonal B cell activation.<sup>(188, 190, 240, 247-249)</sup> Adhesion molecules are also an important component of GC formation. Lymphoid-function associated antigen (LFA-1) and very late activation factor (VLA-4) and their ligands, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) have been shown to be important as well.<sup>(240, 250-253)</sup>

Cytokines such as B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are members of the TNF superfamily and are implicated in GC formation.<sup>(254)</sup> They can be expressed by activated T cells, follicular cells and dendritic cells. INF- $\gamma$  stimulates the expression of BAFF, and it is believed that levels of BAFF must be tightly regulated to maintain B-cell survival without triggering autoimmunity.<sup>(255)</sup> Although the local levels of BAFF are not known, serum levels of BAFF are reported to be higher in SLE, RA and SS.<sup>(256-258)</sup> There is growing evidence that impaired BAFF secretion and the dysregulation of BAFF-mediated processes play a role in the



development of SS.<sup>(258, 259)</sup> It may be true that BAFF is required for maintenance of GCs but not initiation.<sup>(260, 261)</sup>

The cytokines IFN- $\gamma$ , IL-4, IL-10 and INF- $\alpha$  augment BAFF expression in monocytes, macrophages and dendritic cells.<sup>(262)</sup> It is possible, then, that in SS patients with GC, the disproportionate levels of these cytokines may perpetuate BAFF expression in the dendritic cells, monocytes and T cells that would further activate T and B cells.<sup>(255)</sup> Indeed, SS patients do have a distinct set of circulating cytokines and growth factors that are highly upregulated.<sup>(263)</sup>

It is the particular environment of these lymphoid infiltrates that is a risk factor for B cell lymphoma development. It is believed that chronic autoantigen B cell stimulation, mediated by surface Ig receptors, increases the risk for monoclonality and, finally, malignant transformation by a subsequent oncogenic event.<sup>(264)</sup> The malignant B cells seem to be derived from “normal” B cells creating immunoglobins in the inflamed exocrine glands of SS.

#### **2.4.8 The influence of cytokines**

In SS the cytokine milieu changes in the lacrimal and salivary glands, and this may be one of the earliest changes in SS. The local production of these inflammatory cytokines comes from both mononuclear and local epithelial cells.<sup>(265)</sup>

The most prominent cytokines found in SS human salivary glands in two major studies were IL-1, IL-6, IL-10, TGF- $\beta$ , IFN- $\gamma$  and TNF.<sup>(266, 267)</sup> The CD4+ T cells of the salivary gland infiltrates were found to be very active locally and to produce 40x more IL-2, IFN- $\gamma$  and IL-10 than did the peripheral serum T cells.<sup>(266)</sup> The salivary epithelial cells themselves produced 40x more IL-1 $\alpha$ , IL-6 and TNF mRNA than normal epithelial

cells.<sup>(266)</sup> Human salivary gland epithelial cells can be induced to secrete IL-1, TGF- $\alpha$  and IL-6 after stimulation with INF- $\gamma$ .<sup>(268)</sup> This expression would contribute to the ongoing inflammation by stimulating the T cells that in turn can contribute to B cell stimulation.

It is believed that Th1 cytokines such as INF- $\gamma$  and IL-2 as well as IL-10, IL-6 and TGF- $\beta$  are important to the initiation and maintenance of SS inflammation,<sup>(269)</sup> while Th2 cytokines may be involved in the progression of the disease. One study has suggested that an absence of certain cytokines such as TGF- $\beta$  may contribute to the disease process as well.<sup>(270)</sup>

Acinar cells also contribute to the pro-inflammatory environment by secreting IL-1 $\beta$ , an inflammatory cytokine that interferes with neurological mechanisms, thus causing reduced secretions from the gland.<sup>(271)</sup>

#### **2.4.9 Hormones: Internal influences of inflammation**

As discussed in the etiology section, hormones have a very great influence on lacrimal gland function, and their imbalance has been linked to the inflammation of SS. Androgens, estrogens and prolactin are important in maintaining lacrimal gland homeostasis. Prolactin may be the most important hormone in the regulation of self-protein exposure. Lacrimal epithelial cells express mRNA for prolactin and prolactin product. Higher levels of prolactin favour activation and proliferation of B cells<sup>(272)</sup> and T cells.<sup>(273, 274)</sup> It is likely that the lacrimal gland is a very safe place for cryptic epitopes until the hormonal environment is changed so that prolactin is found in high concentrations.<sup>(109)</sup> Dendritic cells that mature in this environment would encourage T cells to develop into Th cells that are not tolerant to cryptic epitopes.

With larger numbers of activated Th cells, there are more inflammatory cytokines expressed that then cause the epithelial cells themselves to secrete more inflammatory cytokines that would in turn promote the survival of activated B cells. These B cells would express Igs to autoantigens such as Ro and La, and at this point the lacrimal gland becomes the source and perpetuation of the SS disease process.

Low concentrations of testosterone also play a role in SS inflammation. Indeed, androgens have been considered a major influence on the health of the lacrimal gland.<sup>(94)</sup> In humans, both androgen receptor mRNA and androgen-binding sites have been localized in the lacrimal gland.<sup>(96)</sup> Androgen stimulation of acinar cells via specific receptor activation has a significant influence on protein synthesis in these cells.<sup>(97)</sup> As such, androgen deficiency may result in the inability of the acinar cells to properly produce and secrete its contents, creating the trafficking chaos that may lead to autoantigen exposure.

In summary, the epithelial cells of the lacrimal gland connect with the conjunctiva and cornea to create the mucosal associated lymphoid tissue that protects the eye from the environment and keeps the internal cells functioning. A good deal of surveillance goes on in the lacrimal gland, with T and B cells travelling around quietly. Dendritic cells pass through and are encouraged to mature as peace-loving cells that go to the lymph nodes and keep regulatory T cells going around and back to the lacrimal gland. Although many of these dendritic cells normally take lacrimal autoantigens with them, they discourage effector T cells from getting too involved with the lacrimal gland proteins. Instead they encourage the maturation of regulatory T cells that keep the lacrimal gland calm.

This harmonious relationship requires a background of calming cytokines that are secreted by the lacrimal gland epithelial cells. A balanced hormonal status also contributes to the calm, especially normal levels of prolactin and testosterone.

If an inflammatory event occurs in the gland or if testosterone concentrations become low and prolactin levels high, there is a change in how dendritic cells mature. Also, if the lacrimal gland is overstimulated, requiring a good deal of IgA to be sent down to the ocular surface, a number of proteins such as Ro and La can be sent out, by accident, through the basal membrane of the cell. This means that dendritic cells can take those self-proteins in and move to the lymph nodes to teach the T cells to recognize them and then return to the lacrimal gland and excite the B cells, which will then express autoantibodies to Ro and La. This eventually leads to both local and systemic inflammation. Now a new atmosphere is present, with pro-inflammatory cytokines being released by the epithelial, B and T cells. This environment promotes the generation and survival of effector T cells, which in turn influence the immunoglobulin expression of B cells. This is Sjogren's syndrome.

#### **2.4.10 The role of apoptosis**

Apoptosis, or programmed cell death, is an ongoing and normal phenomenon in the human body.<sup>(275)</sup> When this self-destruction of cells has altered timing, location, proteolytic processing or number of events at one point, there can be changes in the self-proteins that are presented to dendritic cells in the area. The molecules that are involved in apoptosis and that are used to determine the rate of apoptosis in a gland are Fas and FasL, and these markers have been measured in increased concentrations in the glands of SS patients.<sup>(276)</sup> Also, elevated Bax expression in SS salivary glands have been noted and

may be an indicator of ongoing excess apoptosis of the acinar cells.<sup>(277)</sup> Bcl-2 is upregulated in SS lacrimal glands and may serve to inhibit apoptosis of the lymphocytes of the infiltrates.<sup>(276)</sup>

In summary, the flow of inflammation includes the following steps:

1. A local inflammation is initiated by a virus or other toxin, a slow wearing down of some lacrimal acinar cell function because of hormonal changes, ocular surface distress and/or neurological compromise or the arrival of inflammatory cytokines from the inflamed salivary glands.
2. The inflammation causes a release of pro-inflammatory cytokines by distressed acinar cells and a change in trafficking of self-proteins, leading to their exposure outside the basal membrane. Dendritic cells are likely to pick them up.
3. Acinar cells, because of exposure to the pro-inflammatory cytokines, become APCs as they create MHCII molecules and expose self-proteins such as Ro and La that have now relocated in the cytoplasm because of the confusion within the cell.
4. The formation of HEVs within the lacrimal gland is a response to the cytokine environment. The cytokines, chemokines and cell-adhesive molecules on the HEVs of the gland cause migration and homing of lymphocytes and dendritic cells to the affected gland.
5. Because of the inflammatory environment, local dendritic cells now take up self-antigens and take the message to the lymph nodes to teach Th cells to recognize the self-proteins. The Th cells return to the lacrimal gland to identify Ro and La and stimulate the local B cells to become Ig secreting plasma cells.

6. As time goes on, the lymphocytic infiltrate organizes itself to maintain a constant inflammation. Specific chemokines and cytokines promote the local reproduction and prolonged life of B and T cells. This organization is present in a spectrum from mild organization to actual germinal centres.

The pathophysiology of SS salivary and lacrimal gland inflammation and the subsequent systemic pathology is still in the early stages of understanding. There is, however, a clearer picture of the T, B, acinar and dendritic cell roles in this inflammation and the extraordinary influence of the cytokine and hormonal environment.

## **2.5 The Clinical Presentation of Sjogren-Related Dry Eye**

### **2.5.1 Ocular surface symptoms**

Symptoms of dry, irritated eyes are often the primary complaint in SS, and dryness for at least 3 months is one of the criteria for the diagnosis of Sjogren's syndrome.<sup>(28)</sup> Although no level of intensity is required for patients to meet this criterion, SS patients tend to have more frequent and more severe symptoms of ocular surface irritation compared with other, non-SS dry eye patients. Approximately 80% of SS subjects have reported frequent to constant dryness and discomfort in several studies.<sup>(278-280)</sup> In addition to frequency, there are also qualitative differences in symptoms of dryness.<sup>(279, 281, 282)</sup> Sjogren's syndrome patients commonly describe their eyes as dry, burning, gritty or having a foreign body sensation.<sup>(31, 279, 280, 283, 284)</sup> In comparison, patients with mild to moderate dry eye report dryness, soreness and light sensitivity as their most frequent symptoms.<sup>(285)</sup> Clearly, SS patients have more intense and more frequent dry eye symptoms.

Symptoms of ocular dryness are a result of the stimulation of nociceptors of the ocular surface. Just below the deficient tear film of the SS patient lies the ocular surface and its exquisitely sensitive set of nerve endings.<sup>(286)</sup> The cornea and conjunctiva are innervated by the trigeminal nerve. Corneal sensory nerves derived from the ciliary branch of the ophthalmic division of the trigeminal nerve enter the cornea radially at the mid-stromal level.<sup>(286)</sup> The conjunctival nerves derive from both the ophthalmic and maxillary branches of the trigeminal nerve.<sup>(287)</sup> The cornea has exclusive free nerve terminals, while the conjunctiva has encapsulated nerve endings with higher density in the limbal area.<sup>(288, 289)</sup> Stimuli to the ocular surface excite these nerve endings and send afferent information by the trigeminal nerve to the trigeminal spine nucleus. The nerve bundles then pass through the thalamus to the somatosensory cortex, which is the seat of significant distress in the SS patient.<sup>(290, 291)</sup>

The manner in which the sensory nerve endings fire is complex. However, recent research with the Belmonte pneumatic esthesiometer has allowed researchers to characterize the response of the human ocular surface to chemical, thermal and mechanical stimuli.<sup>(292-296)</sup> The cornea and conjunctiva have many classes of sensory nerve fibres, identified as mechanoreceptors, mechano-nociceptors, polymodal nociceptors and cold receptors.<sup>(297-299)</sup> Mechanical stimulation of the cornea is believed to excite nociceptors, some of which respond to many stimuli. These nerves then are labelled as polymodal receptors or specific mechanoreceptors.<sup>(297, 300-303)</sup> The sensation evoked by chemical stimulation, as studied with CO<sub>2</sub>, is interpreted as stinging or burning and likely represents the firing of polymodal nociceptors or chemonociceptors.<sup>(297, 302-304)</sup>

Numerous factors are present that could enhance sensory ocular surface nerve firing in SS related dry eye. These include significantly reduced tear flow,<sup>(28)</sup> corneal and conjunctival surface changes that are in part observed as staining,<sup>(28, 305, 306)</sup> changes in tear chemistry including increased osmolarity<sup>(307)</sup> and surface inflammation.<sup>(308)</sup> The reduced tear volume on the surface of the eye increases frictional forces when the lid rubs over the ocular surface with the blink, thus enhancing mechanical stimulation of the ocular surface nerves. The increase in tear osmolarity<sup>(307)</sup> may act as a chemical stimulant of surface sensory nerves as would the presence of numerous inflammatory mediators.<sup>(309)</sup> Finally, the increased cell sloughing and subsequent exposure of the corneal and conjunctival nerves is likely to be a factor in SS symptomatology.<sup>(305, 306, 310)</sup> To what degree each of these factors as well as others is responsible for the symptoms of dry eye in SS is not known.

The sensory nerves of SS patients may be altered by the disease. Although we cannot use standard biomicroscope examination to visualize the nerves of the ocular surface, a significant role for aberrant neuronal function in association with SS does appear to exist.<sup>(310)</sup> That corneal nerves have morphological changes in SS has been shown recently through confocal microscopy.<sup>(311)</sup> These SS corneas had fewer sub-basal nerves and higher nerve tortuosity grades that correlated with increased symptoms of dry eye.

Despite a growing understanding of ocular surface sensitivity, there remains a poor correlation between ocular symptoms and conventionally measured signs of ocular surface disease.<sup>(312)</sup> It is clear that a large component of this disease is not yet captured by our clinical examination. Some SS patients who present with severe staining scores and



reduced Schirmer have very few symptoms. There is some belief that over time the cornea and conjunctiva may become desensitized either at the end organ or at more central areas of sensory tissue.<sup>(313, 314)</sup> Conversely, dry eye patients may present with significant symptoms and very few signs. Clinical wisdom surmises that these patients may be responding to chemical or mechanical stimulation at the ocular surface<sup>(309)</sup> that has not yet caused clinically visible surface damage, or that the sensory nerves of these particular patients are hypersensitive.

## **2.5.2 Visual symptoms**

In addition to complaints of ocular surface discomfort, symptoms of reduced visual performance are frequently cited by SS patients. Visual complaints are highly prevalent among dry eye patients in general.<sup>(92, 279, 312, 315)</sup> They describe disturbed vision or blurry, foggy vision that clears temporarily with the blink.<sup>(312)</sup> These transient changes can be profound, resulting in marked drops in contrast sensitivity and visual acuity,<sup>(316)</sup> thus affecting workplace productivity and vision-related quality of life.<sup>(317, 318)</sup>

As the tear film is the first refracting surface of the ocular system, it is not surprising that vision complaints are often a presenting symptom in SS related dry eye. The optical effects of corneal surface irregularity due to epithelial desiccation, tear film instability and evaporation have been visualized and quantified using tools such as corneal topography and wave front analysis.<sup>(319), (320)</sup> An uneven, disrupted tear film in the central cornea appears to be the primary cause of transient vision changes in the SS patient.<sup>(317, 320, 321)</sup>

### 2.5.3 Ocular signs

Tear flow and the condition of the ocular surface are the two observations that are routinely performed in an SS workup. The Schirmer test without anaesthetic is performed first. The Schirmer strips are placed in the temporal third of the lower lid and the patients is asked to close her eyes or blink as normally as possible.<sup>(322, 323)</sup> A result of 5 mm or less in 5 minutes in the worst eye is sufficient to confirm the criterion of signs of dry eye.<sup>(324)</sup>

The second part of the criterion for signs of dry eye is the observation of ocular surface staining.<sup>(28)</sup> At the biomicroscope, staining of the conjunctival nasal and temporal bulbar areas and the cornea as a whole are graded independently on a scale of 0-3 using rose bengal, lissamine green or fluorescein.<sup>(31)</sup>

The ocular surface is severely affected both by the absence of adequate tears and by its own participation in inflammation. Changes in tear quantity affect the ocular surface through increased shear forces of lid-to-surface epithelium. It is generally believed that of all dry eye patients, SS patients have the lowest tear flow and Schirmer scores of 5 mm or less are considered diagnostic in SS.<sup>(28)</sup> It is also suggested that the lack of reflex tearing that is attributed specifically to the SS dry eye contributes to the pathology of the ocular surface that is observed as increased staining.<sup>(325, 326)</sup>

These reduced secretions cause a change in tear osmolarity, and high tear film osmolarity is one of the hallmarks of SS dry eye.<sup>(327)</sup> The effects of high tear film osmolarity are well documented, and it is believed that this tear characteristic contributes to the desiccation of corneal and conjunctival cells in SS.<sup>(307, 328-331)</sup>

## 2.5.4 Tear film quality

The tear film composition is also altered in Sjogren's syndrome. The mechanisms underlying such changes are currently unclear. We do know that regulated proteins are synthesized in the endoplasmic reticulum of the acinar cells, modified in the Golgi apparatus and stored in the secretory granules that fill the apical region of the cell.<sup>(332, 333)</sup> Upon stimulation, the secretory granules fuse with the apical membrane and proteins such as lysozyme, lipocalin and lactoferrin are released into the lumen and incorporated into bulk aqueous flow.<sup>(333, 334)</sup> In SS this orderly process is disrupted and changes in tear composition occur.

Lactoferrin was found to be reduced in SS versus normals in many studies,<sup>(335-338)</sup> although Mackor found increased levels of lactoferrin in SS.<sup>(335)</sup> Lysozyme is reduced<sup>(337, 339)</sup> or unchanged,<sup>(338)</sup> and lipocalin has been found in reduced concentrations in SS compared with both DE and normal tears.<sup>(338)</sup> Pflugfelder et al. noted decreased EGF concentrations in the tears of SS patients.<sup>(309)</sup> Total tear protein is also reduced in SS.<sup>(338)</sup>

Increased levels of certain proteins that are associated with inflammation have been noted in SS versus normals. IL-1,<sup>(340)</sup> IL-6,<sup>(341, 342)</sup> MMP-3,<sup>(340)</sup> serum albumin<sup>(343)</sup> and  $\beta$ 2-microglobulin<sup>(344)</sup> have been identified in the SS tear film.

Mucins constitute an important part of the precocular tear film and ocular surface and play a key role in the retention of water and other tear fluid components on the ocular surface.<sup>(345, 346)</sup> Both secreted (MUC2, MUC5AC, MUC5B, MUC7, MUC19) and membrane bound (MUC1, MUC4, MUC16) mucin forms have been reported to be expressed by ocular surface epithelia.<sup>(347-350)</sup>

Most researchers describe SS as a disease of reduced mucus production and secretion.<sup>(351-353)</sup> Certainly, the secreted mucin MUC5AC has been shown to be reduced in SS tears and conjunctival cells.<sup>(354)</sup>

Transmembrane mucins have also been studied in DE disease.<sup>(349, 355)</sup> Danjo et al. have reported that the conjunctival epithelial cell distribution of H185 (MUC16) is altered in non-SS dry eye subjects.<sup>(355)</sup> Data on other membrane bound mucins has suggested that the expression of mucosal epithelial membrane mucin (as detected by an uncharacterized antibody referred to as AMEM2) is reduced in SS and non-SS dry eyed subjects compared with controls.<sup>(353)</sup> Jones et al. found reduced MUC1 on surface epithelial cells in SS.<sup>(356)</sup> However, at the genetic level some authors have failed to find differences in MUC1 or MUC4 gene expression between controls and SS subjects.<sup>(354)</sup> Part of this thesis describes a study in which MUC16 was found in increased concentrations in the tear film and at the genetic level in conjunctival epithelial cells in SS compared with DE and normal tears.<sup>(357)</sup>

It seems that alterations in mucin production and secretion are a part of SS dry eye. These changes have been linked by some to ocular surface pathology as seen through surface cell vital staining. Alterations in membrane MUC16 has been studied in relation to conjunctival staining. Danjo et al.<sup>(355)</sup> studied non-SS aqueous deficient dry eyed subjects and found a significant correlation between staining scores of the temporal conjunctiva and an altered H185 (MUC16) binding pattern. Two studies used a human corneal-limbal epithelial cell line (HCLE) to demonstrate that MUC16 surface protein protects against rose bengal invasion.<sup>(349, 358)</sup> Others have reported a positive correlation

between decreased transmembrane mucin (not identified) and higher rose bengal staining in aqueous deficient dry eye.<sup>(353)</sup>

### **2.5.5 Conjunctival cells**

The conjunctival epithelium changes in SS. It suffers insult from the lids, reduced supplies of autocrine growth factors and inflammation that can result in changes of cell phenotype. Pflugfelder et al. use impression cytology to study conjunctival cells in SS patients compared with other dry eye subjects and normals.<sup>(359)</sup> They found squamous metaplasia in 89% of samples from the temporal and 60% of the inferior bulbar conjunctiva. This change of phenotype has been reported by others.<sup>(360-362)</sup> Kawasaki et al. did a comprehensive assessment of gene expression in the SS conjunctiva using brush cytology.<sup>(363)</sup> Several genes of keratinization were found to be upregulated as well as IL-6, MIG (a cytokine secreted by monocytes), amphiregulin (an autocrine growth factor), fibronectin, HLA-DR and defensin- $\beta$ 2.

The conjunctiva participates easily in inflammation because it is highly vascularized and contains lymph channels, resident lymph cells and Langerhan cells.<sup>(364)</sup> Inflammatory cells have been shown to gather in the conjunctiva in SS. Of note is the fact that plasma cells<sup>(151, 359)</sup> observed within the normal human conjunctiva were found to be associated with accessory and main lacrimal glands, emphasizing the ease with which SS inflammation might spread from the lacrimal gland to the ocular surface.<sup>(151)</sup> The type of lymphocytic infiltration of the SS conjunctiva has also been studied.<sup>(359, 365, 366)</sup> These inflammatory cells were predominantly T cells with a smaller number of B cells observed.<sup>(366, 367)</sup>

Immunopathological changes of the SS conjunctiva also include higher expression of immune activation markers, HLA-DR and I-CAM, and elevated levels of IL-6, suggesting that the conjunctival epithelium becomes a participant in the inflammatory process.<sup>(309, 366-371)</sup> Pflugfelder et al. found mRNA for several other inflammatory and one anti-inflammatory cytokine in the conjunctival epithelium of SS patients.<sup>(309, 369)</sup> mRNA for IL-1 $\alpha$ , IL-8, TNF- $\alpha$  and TGF- $\beta$  were identified.

Clearly there are many changes in the physiology of the conjunctiva in SS.

### **2.5.6 Corneal cells**

Corneal staining is a part of the diagnosis of SS and is considered an important objective insight into the pathology of that tissue.<sup>(28)</sup> The cornea suffers insults from the lid because of the reduced volume of tears and is affected by alterations in tear film chemistry in SS.

Although the human cornea is an immunologically privileged site, as is witnessed in the high success of allograft transplantation, inflammation can occur and the devastating effects of SS inflammation can be seen in the sterile ulceration of this tissue. This type of inflammation is most commonly associated with rheumatoid arthritis<sup>(372, 373)</sup> and its prevalence in pSS and sSS is not known. The relationship of corneal lysis and SS remains confused in the literature. Bloch et al.<sup>(7)</sup> found 3 of 63 SS patients had corneal ulceration. However RA was part of the definition of SS in this study. Others have found low numbers as well.<sup>(374, 375)</sup>

The ocular surface in SS is greatly changed. Patients present with symptoms of dry eyes and blurred vision. The inflammation of the lacrimal gland reduces the secretions to the ocular surface causing shear force stress and changes in osmolarity that

alter surface cell function. Qualitative changes in the tear film from lack of autocrine proteins and gel forming mucins and the addition of inflammatory proteins also affect the surface cells. The changes in transmembrane mucin expression and the upregulation of inflammatory cytokines within conjunctival epithelial cells add to the pathology of the SS ocular surface.

## **2.6 The Clinical Presentation of Sjogren-Related Dry Mouth**

### **2.6.1 Symptoms of dry mouth**

SS patients will often present with symptoms of dry mouth. They report difficulty in swallowing their food, an inability to speak continuously, changes in sense of taste, a burning sensation in the mouth, an increase in dental caries and an inability to wear dentures well.<sup>(376)</sup> They are less able to eat spicy foods. A useful question that differentiates more severe forms of dry mouth is whether bread or crackers can be eaten without the consumption of water.<sup>(377)</sup>

### **2.6.2 Signs of dry mouth**

Salivary testing can be performed easily. It takes 15 minutes for collection and no stimulation is used. Patients should be fasting and should not have brushed their teeth, rinsed their mouth or smoked tobacco for at least 1 hour prior to the test. The patient is left seated and inclined forward slightly. The saliva is collected in a cup and measured after settling of the froth.<sup>(31)</sup> Flow rates of 1 ml or less per 1 minute are considered diagnostic of dry mouth. The mouth can be examined grossly and typical signs are a loss of papilli and a lack of normal salivary pooling beneath the tongue. SS patients have

increased dental caries and loss of teeth.<sup>(378)</sup> They also experience more frequent angular cheilitis from candidosis.<sup>(379)</sup>

The most common presentation of SS is that of dry eye and dry mouth. A careful history and simple testing of tear flow and ocular surface staining characteristics will help to distinguish those with more severe forms of dry eye and thus potential SS patients.

## **2.7 Systemic Manifestations of SS**

Sjogren's syndrome is a systemic inflammatory disease, which, in addition to its characteristic clinical presentation of dry eye and dry mouth, can involve other tissues and organs. Eye care providers can improve their chances of stratifying their dry eye patients as SS if the systemic manifestations that can present in SS are well-known and reviewed in the history.

Non-specific questions such as the following will help to identify any systemic complications that may be a result of this inflammatory disease.

1. Are you more tired than usual? Have you been running a temperature?
2. Do you have aches and pains in your joints and muscles?
3. Do your fingers get extraordinarily cold and do they turn colour?
4. Do you have rashes or other skin problems?
5. Do you have a persistent cough?
6. Do you have trouble swallowing or digesting your food?
7. Do you have tingling or odd sensations in your hands or feet? Is your balance off?
8. Have you noticed any swelling around your neck?

Systems identified through history and observation include:



1. Musculoskeletal, including fatigue and fibromyalgia
2. Vascular – Raynaud’s phenomenon
3. Cutaneous
4. Pulmonary
5. Gastrointestinal (GI)
6. Neurological
7. Lymphoid
8. Gynecological
9. Maternity

Systemic manifestations requiring invasive testing include:

1. Renal
2. Hematologic

The prevalence of these manifestations is tainted by the population studied, the definition of the manifestation and the interest of the group that is doing the report. Although the pathophysiology of many of these systemic manifestations is unknown, it is believed that the circulating autoantibodies and high levels of gammaglobulins may cause various organs to become inflamed.<sup>(380-382)</sup> However, there is some belief that local changes in the epithelia of the various organs cause lymphocytic infiltration that may be the underlying systemic event.<sup>(383)</sup>

### **2.7.1 Musculoskeletal disease in SS**

#### **A. Myalgia**

Aches and pains and a general sense of illness are often a part of the clinical presentation of Sjogren's syndrome. Myalgia is usually a symptom-based diagnosis, but some studies have incorporated biopsy analysis.

The earliest data come from the Bloch group's research in 1965, in which 62 patients were studied at the National Institute of Health.<sup>(7)</sup> The criterion for SS diagnosis was the presence of at least 2 of the following: dry eyes (filaments, rose bengal staining or Schirmer <15mm), dry mouth (stimulated salivary flow) and rheumatoid arthritis. Myopathy, defined as idiopathic progressive disease of muscle, was present in 4 of 4 patients grouped as Sjogren's syndrome with polymyositis. Of the remaining 58 patients, Bloch reports varying degrees of muscle weakness and atrophy. Muscle biopsies in 8 of 12 (67%) patients who had SS and RA showed mild chronic focal myositis with infiltrates of inflammatory cells. Nine (9) of 23 (39%) patients who had sicca complex alone and no underlying connective tissue disease showed histological evidence of chronic focal myositis.

Bunim did not report a prevalence in 1961 but did describe the myalgias of SS patients as most often proximal, although distal myopathies were reported.<sup>(384)</sup> Using a questionnaire, Al-Hashimi et al. reported that 44.4% of their 169 SS patients reported symptoms of myalgia.<sup>(385)</sup>

More recently, studies have suggested that SS patients often report transient and self-limiting episodes of flu-like symptoms with low-grade fever, fatigue, arthralgias and myalgias.<sup>(386)</sup> Muscle biopsies in SS patients with and without symptoms may demonstrate focal infiltration of the muscles with lymphocytes, making a diagnosis by

history less reliable.<sup>(387)</sup> In fact, the Lindvall study suggested that up to 50% of patients with SS and detectable myositis do not have symptoms.<sup>(388)</sup>

The mechanism of the myalgias is not completely understood. Eriksson et al. in 2004<sup>(389)</sup> reported that pSS patients with myalgia had diminished peripheral blood mononuclear cell cytokine release and an increase in serum IL-18. They suggest that impaired cytokine regulation may have pathogenic importance in the myalgia of pSS.

### B. Arthralgia

Arthralgia is the symptom of joint pain that is often accompanied by tenderness and swelling. This is a relatively common presentation in SS. The diagnosis of arthralgia may come from symptoms or a more rigorous workup involving imaging techniques.

The Bloch group<sup>(7)</sup> reported in 1965 that of the 23 patients with sicca complex and no other autoimmune disease, 39% had a history of current or past joint pain. Three of these patients had osteoarthritis, while two others reported recurrent episodes of arthralgias.

More recently, joint pain was found to be present in approximately 50% of patients in 1983.<sup>(390)</sup> The Bloomsbury study of 66 patients in 1994 confirmed this estimate.<sup>(391)</sup> They found arthralgias to be more prevalent than arthritis. However, none of this series of 66 patients had radiological findings of erosive joint disease. This finding was slightly lower than previous studies where prevalence of 64%-96% has been recorded.<sup>(392-394)</sup> Using a questionnaire, Al-Hashimi found arthralgia in 75.2% of cases in 2001,<sup>(385)</sup> while Garcia-Carrasco et al. in 2002 reported a 37% prevalence in a large cohort of 400 patients.<sup>(382)</sup>

### C. Fatigue and fibromyalgia

Fatigue is one of the major presenting symptoms in SS.<sup>(391, 393, 395)</sup> Barendregt et al. observed that 50% of SS patients suffered from fatigue.<sup>(396)</sup> The mechanism of fatigue is not well understood. There are reports that fatigue is a function of excess cytokines such as IL-1 or TNF-alpha in autoimmune disease,<sup>(397)</sup> although others have found no such correlation.<sup>(398)</sup> Fatigue has many dimensions. There is general fatigue, physical fatigue, reduced activity, reduced motivation and mental fatigue.

There is some confusion in the literature as to the relationship of SS and fatigue because of variables such as depression and fibromyalgia. In 2002 Bax et al.<sup>(399)</sup> reported on 36 consecutive patients with pSS as defined by the revised European criteria (1997) for SS. The comparison groups were 18 patients with secondary SS and 34 healthy controls. They used the Dutch Fatigue scale (DFS) to quantify fatigue. Fatigue was higher in both primary and secondary SS compared with controls. However, the level of depression confounded the results, as 58% of patients with pSS scored high or very high in depression questions. Therefore with further analysis, 79% of the fatigue in pSS could be explained by depression, total level of immunoglobins and thrombocyte counts.

However, questionnaires such as the Medical Outcome Study Short Form 36 (SF-36) and other studies have shown that disabling fatigue is a prominent symptom for many patients with SS.<sup>(396, 400, 401)</sup> Bowman et al.<sup>(402)</sup> and Barendregt et al.<sup>(396)</sup> have shown that fatigue is a prominent factor alone, after eliminating such variables as depression and fibromyalgia. Godaert et al. also concluded that general fatigue was a unique factor in pSS when depression was eliminated as a variable.<sup>(403)</sup>

Fibromyalgia may also be increased in patients with SS.<sup>(404)</sup>

## **2.7.2 Vascular manifestations of SS**

### **A. Raynaud's phenomenon**

Raynaud's phenomenon is a vascular disorder that is characterized by episodes of reversible digital ischemia in response to cold or stress.<sup>(405)</sup>

In the Bloomsbury series, 39% of patients experienced Raynaud's.<sup>(391)</sup> Others have reported anywhere from 16% to 54% of SS patients with this disease.<sup>(7, 394, 406)</sup>

The most recent report is by Garcia-Carrasco et al. from 2002.<sup>(407)</sup> This cross-sectional study was conducted on 320 consecutive patients who fulfilled 4 or more of the European community study group 1993 criteria for pSS. Raynaud's was present in 40 of 320 or 13% of the patients, and all were women.

## **2.7.3 Cutaneous manifestations of SS**

Various cutaneous manifestations of SS have been described, including the following.

### **A. Dryness**

Dryness of the skin is a common symptom in SS. As early as 1965, Bloch reported 33 of 62 (53%) patients in his series as having symptoms of dry skin.<sup>(7)</sup> In 2001 in the USA, dry skin was reported in 55.6% of a population of SS patients.<sup>(385)</sup>

The mechanism of dry skin is still not fully understood. It has been suggested that this dryness may be a result of the decreased capacity for sebaceous gland secretion.<sup>(386)</sup>

A more recent dermatological paper from Italy suggests that xerosis of the skin is a common finding in SS that is not related to reduced sebaceous and sweat gland production but rather a specific change of function in the stratum corneum.<sup>(380)</sup>

### **B. Rash**

Various skin rashes have been reported to be associated with SS. These can be benign but in certain cases represent a severe vasculitis.

When the blood vessels of the extremities become inflamed, a hypergammaglobulinemic purpura results and is usually present symmetrically in the lower extremities. A recent paper by Ramos-Casals et al. in 2004 showed that 10% of 52 pSS patients presented with vasculitis,<sup>(408)</sup> while in the same year Bernacchi found a prevalence of 30%.<sup>(380)</sup> Ramos-Casals described a significant predominance of small vessel (leukocytoclastic) versus medium vessel vasculitis. This life-threatening vasculitis has been closely related to cryoglobulinaemia<sup>(409, 410)</sup> In these cases, it is assumed that immune complexes become trapped at the bifurcation of small blood vessels, leading to complement activation.<sup>(411)</sup>

Another common presentation of the skin in SS is polycyclic, photosensitive cutaneous lesions that had previously been reported solely in Asian patients<sup>(408, 412, 413)</sup> but are now reported more generally. These lesions are clinically identical to those in patients with subacute cutaneous lupus erythematosus.<sup>(116)</sup>

Also patients may present with erythema multiforme, a painful red rash of the lower legs.<sup>(391-393, 414)</sup>

#### **2.7.4 Pulmonary manifestations of SS**

SS patients may report dry throats, chronic coughs and episodes of bronchitis and pneumonia. As early as 1965, Bloch reported on the variety of pulmonary presentations in SS. Thirty-seven (37) of 62 patients (60%) reported nasal dryness.<sup>(7)</sup> Lower respiratory tract disease was also noted in this group. A history of pleurisy was reported in 2 patients and 2 others showed pleural thickening. Another 2 patients had been admitted to hospital

in the past for bronchitis or pneumonia. Eight other patients reported non-hospitalized diagnoses of bronchitis or pneumonia. In all, 14 (22.6%) of these 62 patients had lower respiratory disease at some time on their course of SS. Lung disease has been commonly reported by others in SS.<sup>(15, 415)</sup>

It is believed that lung disease occurs early in the process of SS and most often does not progress.<sup>(416)</sup> However, SS patients are also known to develop pleurisy and interstitial lung disease with lymphoid interstitial pneumonitis.<sup>(392, 417, 418)</sup>

Fox suggests that the lack of secretion from exocrine glands of the upper respiratory tract causes the nasal and bronchial dryness.<sup>(377)</sup> Desiccation of the tracheobronchial tree is a common respiratory manifestation that is also a result of diminished secretions of the local mucous glands.<sup>(419)</sup> The dryness of the trachea manifests itself with a cough that may be mild or strong. Sjogren himself described this phenomenon and it was labelled “bronchitis sicca” by Alarcon-Segovia in 1978.<sup>(420)</sup> Al-Hashimi et al. in 2001 stated that 9.5% of their SS patients had a chronic cough and 14.8% reported recurrent bronchitis.<sup>(385)</sup>

Recent studies have described the predominance of bronchial/bronchiolar involvement rather than interstitial disease.<sup>(421, 422)</sup> Papis et al. suggested that small airway disease is the primary disorder.<sup>(423)</sup> Franquet et al. in 1999 found bronchiolar abnormalities in 33% of their patients,<sup>(424)</sup> while Taouli et al. found large or small airway disease was seen in more than 50% of their patients.<sup>(425)</sup>

### 2.7.5 Gastrointestinal (GI) manifestations of SS

SS patients may present with problems of digestion and weight loss. Dryness of the mouth and esophagus that results from a lack of saliva is believed to be the main cause of this condition.<sup>(426, 427)</sup>

Esophageal disturbance was noted in 3 patients in the Bloch series but was found in the group with the concomitant diagnosis of progressive systemic sclerosis.<sup>(7)</sup> Others have described decreased esophageal motility<sup>(428,429)</sup> that may be a function of reduced secretions of the esophageal glands or a myositis.<sup>(430)</sup>

Acid reflux and heartburn were also reported as a common presentation in SS patients.<sup>(431)</sup> They are likely a result of the increased levels of gastric acid that are not neutralized by the saliva. The gastric mucosa of these symptomatic patients looks atrophic. Biopsies can reveal cellular infiltrates that consist mainly of T lymphocytes and are thus reminiscent of salivary and lacrimal gland infiltrates.<sup>(432)</sup> Al-Hashimi et al. reported in 2001 that reflux, indigestion, diarrhea and constipation were present in 20.1%-29.6% of their SS population.<sup>(385)</sup>

In later studies, Fox et al. found that the liver and pancreas of the upper gastrointestinal tract were the most common organs to be affected in SS.<sup>(386)</sup> It seems likely that the secretory nature of the pancreas makes it a target for inflammation in SS and that the liver may be subject to the same stresses that occur in other autoimmune diseases. Clinical and biochemical evidence of liver disease is found in 5%-10% of SS patients. Primary biliary sclerosis (PBC) is also a common presentation in SS patients.<sup>(386, 392)</sup>



### 2.7.6 Neurological manifestations of SS

SS patients may report tingling of the hands and feet or disturbances of balance and cognition. Thus the neurological complications of SS may be peripheral or central.<sup>(433)</sup>

The neurological presentations of SS can mimic both SLE and MS.<sup>(426, 434)</sup> For some time, SS patients were closely compared neurologically with patients with multiple sclerosis.<sup>(414)</sup> In fact, MS was considered to be the main imitator of CNS-SS disease, rendering the distinction between the two entities quite difficult in some cases.<sup>(435)</sup> There are however, some researchers such as Simmons-O'Brien et al. who suggest that there are strong differences.<sup>(436)</sup>

There is little mention of neurological manifestations of SS in the early literature. In the later studies there is a good deal of variability in the reported percentages of SS patients presenting with neurological involvement. This variability is a result of the various diagnostic criteria that are used to determine the presence of neurological complications as well as the population base that is studied.<sup>(437)</sup>

The type of neurological involvement is also varied. It includes focal deficits such as hemiparesis, hemisensory deficits, seizure disorders and movement disorders, and more diffuse disorders such as headache, cognitive dysfunction and psychiatric abnormalities.<sup>(438)</sup> SS patients may have central nervous system involvement, peripheral neuropathy or cerebrospinal fluid abnormalities.

The percentage of SS patients who do have neurological complications is not well-known but studies have suggested numbers as high as 25%,<sup>(439)</sup> although these percentages have been questioned.<sup>(437, 440)</sup> One stringent study that followed 100 patients

suggested a 20% prevalence. Patients were noted to have clinical manifestations, spinal fluid abnormalities and MRI abnormalities similar to those seen in MS.<sup>(436)</sup>

In 2001 Barendregt et al.<sup>(438)</sup> followed 39 consecutive pSS patients in a clinic in Rotterdam. They used questionnaires, physical examination, quantified sensory neurological examination and nerve conduction studies to determine the prevalence of peripheral neurological involvement in asymptomatic pSS patients. They found 21% of patients were symptomatic while abnormal neurological examination results were found in only 18%.

The types of lesions were well described by Delande et al. in 2004,<sup>(441)</sup> when they did a retrospective study on 82 patients with pSS and neurological disease in France. They used the new 2002 American-European criteria for pSS. Fifty-six of 82 or (68%) of patients had CNS disorders that were focal or multi-focal. Twenty-nine patients had spinal cord involvement. Thirty-three patients had brain involvement and 13 patients had optic neuropathy. They also reported diffuse CNS symptoms: 7 had seizures, 9 had cognitive dysfunction, and 2 had encephalopathy. Fifty-one patients had peripheral nervous system involvement (PNS). This included most frequently symmetrical axonal sensorimotor polyneuropathy with a predominance of sensory symptoms or pure sensory neuropathy (28 patients), followed by cranial nerve involvement affecting trigeminal, facial or cochlear nerves (16 patients). Of the 58 patients who had MRIs, 70% presented with white matter lesions and 40% met the criteria for MS.

One of the most common presentations of peripheral neuropathy in primary SS is that of the feet and hands in patients with hypergammaglobulinemia purpura. This polyneuropathy affects the lower extremities more than the upper. The symptoms are

usually of anesthesia and paresthesia in a stocking/glove distribution but are usually mild and not disabling.<sup>(391, 392, 442, 443)</sup>

There are also mixed polyneuropathies that have stronger symptoms of painful paresthesia. If motor involvement predominates, the patients will demonstrate motor dysfunction such as mononeuritis multiplex, which is often associated with vasculitis.<sup>(443)</sup>

Cranial nerve involvement in SS may be peripheral or central. The most common presentation is trigeminal sensory neuropathy.<sup>(444)</sup> Less frequently seen are facial nerve involvement, sensory hearing loss, peripheral vestibular dysfunction and III, X and XII cranial neuropathies.

The pathogenesis of these neuropathies is not well understood. Alexander et al. in 1986 reported on a possible immune mediated mechanism of CNS disease in SS.<sup>(445)</sup> He analyzed the cerebrospinal fluid of SS patients with active disease and found evidence of lymphocytosis, raised IgA index and one or more oligoclonal bands on electrophoresis. This suggests that lymphocytes have migrated to the CNS and are synthesizing antibodies intrathecally. Therefore CNS disease in SS may be immunologically mediated.

Limited evidence suggests that peripheral neuropathies are secondary to small vessel mononuclear inflammatory and ischemic/hemorrhagic vasculopathy in the epineurium or vasa vasorum.<sup>(434)</sup> When pure sensory neuropathy is observed clinically, evidence of lymphocytic infiltration of the dorsal root ganglion has been observed.<sup>(446)</sup> There was suggestion that these infiltrates contained activated cytotoxic T cells. Thus the brain stem lesion reflects somewhat the lesions of the salivary and lacrimal glands.

### 2.7.7 Lymphoproliferation in SS

One of the important reasons for identifying SS patients is their increased risk for lymphoma, which usually presents as marginal-zone B cell lymphoma of the mucosa-associated lymphoid tissue (MALT), a form of non-Hodgkin lymphoma (NHL).<sup>(447, 448)</sup> The most recent meta-analysis suggests a high risk for SS with a standard incident rate of 18.8, in contrast to RA and SLE where the incident rate is 3.9.<sup>(449)</sup>

At the clinical level, identifying those at most risk is important. Patients with a history or the presence of swollen glands, particularly the parotid glands, are at a higher risk for this SS complication, as are those with purpura or vasculitis and those with low complement values.<sup>(410, 450, 451)</sup>

Although the mechanism that underlies this higher risk in SS is not known, it is believed that the characteristic B cell dysregulation and hyperactivity of this disease are the important risk factors.<sup>(452)</sup> Most of the lymphomas develop during antigen activation or the post-antigen exposure stages of B cell differentiation.<sup>(453)</sup> It is believed that the lymphocytic infiltrate, particularly in its most organized state of GC, is the place where B cells could become malignant. During the GC high rate of B cell activity and transformation, chromosomal translocations and mutations or regulatory non-Ig genes may occur, leading to cell-cycle deregulation and inhibition of apoptosis.<sup>(454, 455)</sup>

The other form of lymphoma is the more aggressive diffuse large B cell lymphoma. It is the most common form of non-Hodgkin lymphoma in the general population.<sup>(448)</sup>

Kassan et al. followed 136 women with SS for an average of 8 years ending in 1978 to find a 40-fold higher risk for lymphoma than in a normal population.<sup>(456)</sup>

However, there is a great deal of disagreement with this estimate.<sup>(391, 392, 394, 457)</sup> These differences relate, in part, to the difficulty of distinguishing lymphoma from extensive infiltration of benign lymphocytes or pseudolymphoma.<sup>(458)</sup> This pseudolymphoma presents with lymphadenopathy and night sweats but also with biopsies that show lymphocytic infiltration without malignant transformation.

A more recent paper that attempts to estimate the risk for lymphoma is that of Sutcliffe et al. in 1998.<sup>(451)</sup> Seventy-two patients with pSS were studied. Five patients (7%) developed a distinct non-Hodgkin lymphoma of mucosa-associated lymphoid tissue. A history of swollen salivary glands, lymphadenopathy and leg ulcers predicted lymphoma. Others have found a prevalence of 4%-8%.<sup>(459, 460)</sup>

The most recent paper that determines the risk of lymphoproliferative disease in SS is that of Theander et al. in 2006.<sup>(450)</sup> Five hundred and seven Scandinavian patients diagnosed by the most recent AECC with pSS were followed for a median of 8 years. The researchers determined a 16-fold increased risk for development of non-Hodgkin lymphoma. They showed that CD4+ T-lymphocytopenia is a strong risk factor for the development of this disease, as are the previously reported skin vasculitis, low complement factor C3, low C4 and a low CD4+/CD8+ T-cell ratio.

The lymphomas that do occur are predominantly clonal non-Hodgkin B-cell tumors located in the parotid gland, salivary gland and cervical lymph nodes. They have also been located in the stomach, lung and genital mucosa.<sup>(447, 461-463)</sup>

These lymphoma patients require the earliest possible diagnosis and treatment that now is offered with B cell depletion therapies. Lifelong follow-up is required, as there is a high rate of relapse in patients with MALT lymphomas.<sup>(464)</sup>

### **2.7.8 Gynecological manifestations of SS**

Dryness of the eyes and mouth is often accompanied by dryness of the vagina in SS. The Bloch group reported 20 of 62 or 32% of patients had dryness of the vulva or vagina.<sup>(7)</sup> Some of this may have been a function of menopause. In 1961 Bunin also described dyspareunia in SS patients that was a function of reduced secretions of the vagina.<sup>(386)</sup> Vaginal dryness was reported in 38.5% of 169 SS patients in the USA, and this was a significantly higher percentage than that found in their age-matched control group.<sup>(385)</sup>

### **2.7.9 Maternity**

SS patients may report a history of miscarriage. Pregnant SS patients have a higher frequency of the fetal complication of congenital heart block that relates to the transplacental passage of autoantibodies to Ro and La. In some patients recurrent miscarriages or vascular thrombosis are a result of antiphospholipid antibodies.<sup>(465)</sup>

### **2.7.10 Renal manifestations of SS**

SS patients may report frequency of urination. However, most SS associated renal disease has no presenting symptoms.<sup>(466)</sup>

The most common finding in SS is the inability to acidify the urine in response to an administered acid load. It is believed that this is a dysfunction of the distal nephron and may present in 20%-73% of patients.<sup>(386, 393, 394, 467, 468)</sup>

The Bloch study noted “many” patients who had low urine specific gravity in the morning but found no overt renal disease.<sup>(7)</sup>

In the past the renal disease reported in pSS was described as predominantly tubular. More recently, glomerulonephritis has also been described in a number of patients with pSS.<sup>(466, 469-471)</sup>

Tubular and glomerular renal disease have different clinical and prognostic implications. Tubulointerstitial nephritis is a specific tubular epithelitis that is found most often in younger SS patients and is characterized by an indolent subclinical course without development of renal failure.<sup>(472)</sup> In contrast, glomerulonephritis is a severe manifestation of pSS that is closely associated with cryoglobulinaemia and hypocomplementaemia that appears late in the course of SS and is associated with higher morbidity and mortality.<sup>(471)</sup>

### **2.7.11 Hematologic manifestations of SS**

The diagnosis of SS requires serum analysis. Most SS patients have serum antibodies to Ro and La as well as other circulating anti-nuclear antibodies. Several other blood abnormalities have been reported in SS.

Leukopenia, defined as WBC count less than 4000 cells/ml, was present in 20% of SS patients in one study.<sup>(386)</sup> Previous reports have noted leukopenia in 6%-33% of patients.<sup>(457)</sup> The mechanism of this leukopenia is not well understood but could be the result of immune destruction of leukocytes, splenic sequestration, abnormal bone marrow maturation or an accumulation of white blood cells in the organs of inflammation.

In a large series of 400 patients, Ramos-Casals et al. in 2002<sup>(473)</sup> reported cytopenia in 33% of cases, raised erythrocyte sedimentation rate in 22% and hypergammaglobulinemia in 22%.

### 2.7.12 Less common systemic manifestations

Although overt cardiac disease is not reported in SS, echocardiograms do show a high frequency of pericarditis and diastolic dysfunction. Pericarditis was found in 33% of pSS patients by Rantapaa-Dahlqvist.<sup>(474)</sup> Mita et al. found 55.5% of pSS patients to have abnormal echocardiograms,<sup>(475)</sup> while Manganelli et al. found only the E wave was significantly different in pSS patients.<sup>(476)</sup>

Sleep disturbances are a common presentation in SS but no values as to prevalence have been established. The mechanism may be related to cytokine concentrations in the CNS<sup>(398)</sup> that are influenced by SS cytokines in the blood.<sup>(477)</sup>

Headaches, particularly migraine headaches, were found in 46% of patients with SS and only 11% of controls.<sup>(478)</sup> The suggestion was that small vessel pathology may underlie this process.

Thyroid dysfunction was found in 30% of pSS patients and only 4% of controls in France.<sup>(479)</sup>

**Table 2.2: Prevalence of Systemic Manifestations in Sjogren’s Syndrome**

Syst Man	% reported	Author	Date
<b>Musc/Skel</b>			
Myalgia	44.4%	Al-Hashimi et al.	2001 <sup>(385)</sup>
Arthralgia	37%-96%	Shearn	1971 <sup>(480)</sup>
		Pavlidis et al.	1982 <sup>(6)</sup>
		Castro-Poltronieri	1983 <sup>(390)</sup>
		Montecucco et al.	1989 <sup>(481)</sup>



		Vitali et al.	1991 <sup>(393)</sup>
		Moutsopoulos et al.	1993 <sup>(394)</sup>
		Al-Hashimi et al.	2001 <sup>(385)</sup>
		Garcia-Carrasco et al.	2002 <sup>(382)</sup>
Fatigue	50%	Barendregt et al.	1998 <sup>(396)</sup>
<b>Vascular</b>			
Raynaud's	16%-54%	Bloch et al.	1965 <sup>(7)</sup>
		Shearn	1971 <sup>(480)</sup>
		Montecucco et al.	1989 <sup>(481)</sup>
		Youinou et al.	1990 <sup>(406)</sup>
		Skopouli et al.	1990 <sup>(482)</sup>
		Vitali et al.	1991 <sup>(393)</sup>
		Moutsopoulos et al.	1993 <sup>(483)</sup>
		Garcia-Carrasco et al.	2002 <sup>(407)</sup>
<b>Cutaneous</b>			
Dry Skin	53%	Bloch et al.	1965 <sup>(7)</sup>
Rash	5%-30%	Kausman et al.	1994 <sup>(391)</sup>
		Ramos-Casals et al.	1998 <sup>(484)</sup>
		Al-Hashimi et al.	2001 <sup>(385)</sup>
		Garcia-Carrasco et al.	2002 <sup>(382)</sup>
		Ramos-Casals et al.	2004 <sup>(408)</sup>
		Bernacchi et al.	2004 <sup>(380)</sup>

<b>Pulmonary</b>	14.8%-50%	Bloch et al.	1965 <sup>(7)</sup>
		Franquet	1999 <sup>(424)</sup>
		Al-Hashimi et al.	2001 <sup>(385)</sup>
		Taouli et al.	2002 <sup>(425)</sup>
<b>GI (various)</b>	5%-21%	Bloch et al.	1965 <sup>(7)</sup>
		Pavlidis	1982 <sup>(6)</sup>
		Fox	2002 <sup>(386)</sup>
Liver disease	7%	Skopouli et al.	1994 <sup>(485)</sup>
<b>Neurological (various)</b>	20%-68%	Andonopoulos	1990 <sup>(439)</sup>
		Simmons-O'Brien	1999 <sup>(436)</sup>
		Barendregt	2001 <sup>(438)</sup>
		Delalande et al.	2004 <sup>(441)</sup>
		Lopate et al.	2006 <sup>(433)</sup>
Lymphoma	7%	Sutcliffe et al.	1998 <sup>(400)</sup>
<b>Gynecology</b>			
Vaginal dryness	32%-38.5%	Bloch et al.	1965 <sup>(7)</sup>
		Al-Hashimi et al.	2001 <sup>(385)</sup>
<b>Renal</b>	20%-73%	Pokorny et al.	1989 <sup>(467)</sup>
		Vitali et al.	1991 <sup>(393)</sup>
		Moutsopoulos et al.	1993 <sup>(394)</sup>
		Fox	2002 <sup>(386)</sup>

		Ren et al.	2008 <sup>(468)</sup>
<b>Hematology</b>			
leukopenia	20%	Fox	2002 <sup>(386)</sup>
cytopenia	33%	Ramos-Casals	2002 <sup>(473)</sup>
High SED	22%	Ramos-Casals	2002
hypergamma	22%	Ramos-Casals	2002

Abbreviations: Syt Man is systemic manifestations, Musc/Skel is musculoskeletal, GI is gastrointestinal, SED is sedimentation rate, hypergamma is hypergammaglobulinemia.

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### **3 Factor and Regression Tree Analysis of Dry Eye Patients: The Importance of Conjunctival Staining**

#### **3.1 Overview**

**Purpose:** To compare the clinical presentation of 231 patients with primary Sjogren's syndrome (pSS) and 58 patients with secondary Sjogren's syndrome (sSS) with 89 aqueous deficient dry eye (keratoconjunctivitis sicca, KCS) patients, to determine those procedures that best differentiate these groups in the eye care clinic.

**Methods:** The records of all patients seen at the University Health Network Sjogren's Syndrome Clinic from October 1992 to July 2006 were reviewed and documented. Patients were diagnosed as primary and secondary SS by the American European consensus criteria (AECC) of 2002. Those patients seen at the clinic who were not diagnosed with SS and who had symptoms of dry eye and Schirmer scores of  $\leq 10$  mm in 5 minutes in at least one eye were included as aqueous deficient dry eye controls (KCS). There were 90 variables used in the analysis of the total database. A factor analysis was performed using the pSS database. Recursive partitioning was used to create several classification trees that demonstrated which characteristics best distinguished the three groups from each other and particularly pSS from KCS.

**Results:** Factor analysis resulted in 3 factors: Factor 1, corneal fluorescein staining; Factor 2, conjunctival rose bengal staining; and Factor 3, serum Ro and La results. Recursive partitioning of the full database demonstrated that the serum immunoglobulin Ro

and the status of the salivary gland biopsy are most important in distinguishing SS and KCS. The presence of rose bengal staining of the temporal conjunctiva was the most important non-invasive ocular variable to separate the groups. Total rose bengal staining also improved the sensitivity. However, using non-invasive techniques only, staining of the temporal conjunctiva and severity of dry mouth symptoms proved to be the major factors in distinguishing SS from KCS.

**Conclusions:** Corneal and conjunctival staining appear to have clinically complementary yet separate value in pSS dry eye disease. Rose bengal staining of the ocular surface is an important observation in the detection of SS and the differentiation of SS and KCS.

## 3.2 Introduction

Sjogren's syndrome is a systemic autoimmune disease that presents in eye care offices with symptoms of dry eyes. It is important to differentiate SS patients from those with other forms of dry eye disease, as they have systemic complications associated with the disease and a forty times greater risk of MALT lymphoma.<sup>(1)</sup>

The American European Consensus Criterion (AECC) for SS has become the gold standard for the diagnosis of SS.<sup>(2)</sup> This standard does require a positive antinuclear antibody test and/or a positive salivary gland biopsy result. These two critical diagnostic tests are both invasive and expensive.

Since the eye is so much a part of this autoimmune disease, we asked the question: Can SS-related dry eye be differentiated from aqueous deficient dry eye in the eye care clinic through non-invasive testing?

## 3.3 Materials and Methods

Ethics approval was received from the University Health Network research ethics board for this study. A review of charts of those patients seen at the University Health Network Sjogren's Syndrome Clinic from its inception in October 1992 to July 2006 was completed. Patients were diagnosed as primary SS or secondary SS by the American European consensus criteria (AECC) of 2002. KCS patients were those with symptoms of dry eye and Schirmer scores of  $\leq 10$  mm.

Statistical analysis included factor analysis<sup>(3)</sup> of the pSS group (n=231) and recursive partitioning,<sup>(4)</sup> using the R statistical analysis language,<sup>(5)</sup> of the entire cohort (n=378). Factor analysis was used to determine if there were clinical tests that could be

grouped to diagnose pSS. Recursive partitioning, using the R package “rpart,” was used to create classification trees that demonstrated which characteristics best distinguished SS from KCS.

### **3.3.1 The procedures of the SS clinic**

Patients were seen on the same day by all practitioners and laboratories. Four patients were evaluated on one clinic day at the Toronto Western Hospital, in “shotgun” fashion, so that at 9:00 a.m. each of the patients was seen by an optometrist/ophthalmologist or a rheumatologist or a lab technician for blood drawing or an X-ray technician. By noon each patient had attended all 4 stations. In the afternoon they saw the dental surgeon and otolaryngologist in the associated nearby Toronto General Hospital. The clinical testing regimen was determined by each discipline and followed the standard of clinical care for Sjogren’s syndrome patients.

All reports were kept by the rheumatologist and blood work and X-ray findings as well as the pathology report were added to the file during the next weeks. In one month’s time all of the parties met to review the cases, and a decision on the diagnosis was made.

Prior to the SS Clinic visit, all patients received a package of information and forms to fill out. They listed their various health care providers and demographics, and completed a health information form that included diagnoses, medications and health history. There was also a dryness questionnaire that is described below.

### **3.3.2 The ocular examination**

Patients completed a dryness questionnaire prior to their visit to the hospital and brought that information with them. The paper asked:

1. Have your eyes been dry for at least 3 months?
2. Do you use eye drops at least 3 times a day?

Then they were asked to mark a visual analogue scale that represented a range of dryness levels from 0 to 10 on a horizontal line, 100 mm in length, anchored by verbal descriptors at each end and marked by numbers 1 through 10 in equal units. The left end of the line indicated “not dry at all,” whereas the right end represented the highest level, or “extremely dry.” The patient marked the line at the point that best represented his/her perception at that moment, and the degree of symptomatic dryness was scored by observing the number of the line marked, allowing a 0.5 addition if the mark fell in between numbers.

The dry mouth questionnaire followed the same format.

Patients were seen in the eye clinic, and a verbal history was taken concerning their general health and eye status. Of particular interest was any ocular condition such as allergy that might have interfered with the testing.

A Schirmer 1 tear test was done to begin the testing. Patients were seated comfortably and the Schirmer strips were inserted onto the lower lid, approximately 1/3 from the temporal canthus of each eye. Patients closed their eyes comfortably for 5 minutes, at which point the strips were removed and the amount of wetting was recorded using the scale on the strip.

Slit lamp examination with white light was performed next. The general status of the ocular surface was noted. Fluorescein strips were then wetted with non-preserved saline and excess fluid was flicked off. The lower lid of the right and then left eye was lowered and the strip was touched to the tarsal conjunctival surface. The slit lamp

examination was resumed using a cobalt blue filter. The cornea was examined for staining and a diagram was drawn to indicate which areas of the cornea were stained.

This was followed by a drop of topical anaesthetic, Alcaine, applied to each eye on the inferior lid. The patient was asked to close his/her eyes for 30 seconds. Then one drop of liquid rose bengal prepared in the hospital pharmacy was inserted onto the lower lid of each eye. Slit lamp examination was resumed and, using white light, a grading was done of the temporal bulbar, cornea and nasal bulbar areas, graded on a 0-3 scale and recorded in the file.

The results of the assessment were related to the patient and recommendations for treatment were given. The patient then moved on to the next station.

### **3.3.3 The rheumatology examination**

The rheumatologic examination was the longest visit of the day. A complete history and full systems review was performed. A complete physical examination was performed. Some of the relevant features discussed with the patient are listed:

The clinical manifestations were evaluated as follows:

1. Musculoskeletal:
  - a. Myalgia: history of morning stiffness, muscular weakness or muscular pain.
  - b. Arthralgia: history of joint pain, morning stiffness, swelling or deformity.
2. Vascular:
  - a. Raynaud's phenomenon: "history of intermittent episodes of blanching of the fingers at least to the dip joints, with clear

demarcation or a history of at least two colour changes, such as cold induced cyanosis, followed by hyperemia, again with clear demarcation.

3. Cutaneous:
  - a. Dry skin: patient report in history.
  - b. Skin rash/vasculitis: non-blanchable purple spots on the lower extremities, coming in crops. Or observation of non-blanchable purpura or post-purpuric pigmentation.
  - c. Alopecia: patient report of thinning of hair or observation of same
  - d. Pruritis: patient report of itchiness
  - e. Photosensitivity of skin: patient report of sun induced rash, or observation of rash pattern compatible with subacute cutaneous lupus.
4. Pulmonary manifestations: cough, shortness of breath or observation of basal crackles, or abnormal interstitial fibrosis on chest X-ray.
5. GI manifestations: patient report
  - a. Dyspepsia: patient report of post-prandial heartburn.
  - b. Dysphagia: patient report of food sticking half way down.
6. Neurological manifestations: patient report of numbness or finding of lost sensation to at least one modality in a peripheral distribution.
7. Lymphoma: patient report or documentation on physical examination of unexpected glandular nodule or lymphadenopathy, confirmed by

immunohistological markers and polymerase chain reaction studies of DNA.

8. Fatigue: history of unusual and prolonged fatigue without apparent cause
9. Fibromyalgia: by 1990 American College of Rheumatology Criteria – 11 of 18 tender points and a history of pain above and below the diaphragm, and on the right and left sides of the body.
10. Gynecology:
  - a. Vaginal dryness: patient reported in history
  - b. Dysparunia: patient reported in history
11. Maternity: history of recurrent unexplained fetal loss (3 or more miscarriages in the first trimester).
12. Associated systemic diseases: all such diagnoses were confirmed by the rheumatologist on history and/or laboratory investigation.
  - a. Hypothyroidism: history of thyroid supplement use, the finding of elevated TSH or evidence of anti-thyroid antibodies.
  - b. Diabetes: by history or blood work
  - c. PBC: by history, or on laboratory evidence of elevated antimitochondrial antibodies plus liver biopsy and abnormal liver function tests characteristic of this disease.
  - d. Rheumatoid arthritis: by 1997 Revised American College of Rheumatology criteria (Arthritis and Rheumatism 1998).
  - e. SLE: by 1997 Revised American College of Rheumatology Criteria.



- f. Scleroderma: by 1980 American College of Rheumatology Criteria.
  - g. Mixed connective tissue disease: anti-RNP positive with features of more than one connective tissue disease.
13. Renal manifestations: Tubular acidosis: Bicarbonate value of 22mM/L or less in association with a neutral urinary pH.
14. Haematology: Immunology screen: full blood workup in laboratory including detection of antinuclear antibodies (ANA) using indirect immunofluorescence procedure on a Hep-2 substrate (Hep 2000, Immunoconcept, Sacramento, CA), anti-SSA (Ro) and anti-SSB (La) autoantibodies using enzyme-linked immunosorbent assay (Diastat, Bouty, Milan, Italy) and rheumatoid factor (RF) using immunonephelometry (Dade Behring, Newark, DE); evaluation of erythrocyte sedimentation rate (Westergren) and C-reactive protein level
15. X-ray of hands, wrists and chest done routinely looking for erosions, calcinosis or interstitial fibrosis.

### **3.3.4 The otolaryngology examination**

In the otolaryngology clinic, a complete inspection of the mucous membranes of ears, nose, mouth and throat was performed. A minor salivary gland biopsy was undertaken after informed consent.

The otolaryngologist used a minimally invasive minor salivary gland biopsy using the smallest incision required to collect the glands. All minor salivary gland biopsies were performed by an otolaryngologist from the SS Clinic. In his rare absence a qualified

otolaryngology Fellow would perform the procedure. The salivary glands were located by palpation of the lower lip; topical anesthesia (Hurricane) was applied and after 1 minute the lower lip was then infiltrated in the midline with approximately 1-2 cc of 2% xylocaine with 1:1000 epinephrine. After allowing a suitable time for effect, a chalazion retractor was applied to the lower lip and tightened for hemostasis. A small incision (10-15mm) was made with a #15 blade scalpel on the inner surface bordered by the retractor arch. The gland typically bulged from the incision such that they could easily be removed with biopsy forceps. The incision was closed with 4-0 interrupted absorbable sutures and an ice pack was applied for a few minutes. The patient was then able to rinse his or her mouth out with normal saline. The patient was advised that the sutures would fall out on their own in a few days, and that if there were any problems, they would be reviewed in the otolaryngology clinic. In our study this was sufficient to provide adequate glandular lobules for the histopathologic study (4-5 lobules).

### **3.3.5 The histopathology procedures**

All tissue samples were examined by the same pathologist. Briefly, samples were fixed in 10% neutral buffered formalin, processed and embedded in paraffin according to standardized laboratory methods.

Sections measuring 4  $\mu$ m in thickness were cut onto glass slides. In each case three sections were obtained at 200  $\mu$ m intervals. The slides were then stained with hematoxylin and eosin. All samples were analyzed by the same pathologist (DB), who was blinded to clinical and laboratory data. For each patient, the number of glands was counted and the total surface area of the glands was measured, using a microscope equipped with an eyepiece containing a graticule scale (Leica). Lobules with acinar

atrophy and diffuse fibrosis were excluded. Lymphocytic foci were scored according to the method of Greenspan et al.<sup>(6)</sup> Scoring of the lymphocytic infiltrates was performed on each section, according to Greenspan et al.<sup>(6)</sup> and Daniels.<sup>(7)</sup> A Focus Score of 1 per 4 mm<sup>2</sup> was considered abnormal.

### **3.3.6 The dentistry evaluation**

A complete inspection of the mouth, tongue and palette was conducted. The following observations were recorded.

Diagnosis of oral features:

1. Stimulated salivary flow
2. Unstimulated salivary flow
3. DMF
4. Examination of oral mucosa
5. Examination of tongue
6. Candidiasis
7. Other

### **Objective measurements**

The clinical evaluation of the patients was performed according to Navazesh et al.<sup>(8)</sup> Dryness and cracking of the corners and/or the vermilion borders of the lips were noted as was the presence of angular cheilitis, redness or fissuring at the commissure.

Dry tongue blades were used to retract the buccal mucosa bilaterally, and the mucosa was scored as 0 (normal), 1 (looks dry but tissue does not stick to the tongue blade), 2 (looks dry and tissue sticks to the tongue blade), or 3 (looks dry, tissue sticks to

the tongue blade, and the location of one or both parotid ducts is not apparent). A tongue was scored positive for sticking if either or both cheeks stuck to the blade.

Saliva that had accumulated on the floor of the mouth was referred to as the salivary pool and was noted as present or absent.

Non-stimulated and stimulated, whole salivary flow rate measurements were taken. The measurements were performed between 1:00 and 3:00 p.m. by spitting method. Subjects were instructed to refrain from food and beverages for 2 hours before test session. Before salivary collections began, each subject rinsed thoroughly several times with de-ionized water and rested for 5 minutes. The subjects were asked to bend their heads forward and, after an initial swallow, to allow saliva flow into the mouth. Subjects expectorated the saliva into a test tube once per minute, for 10 minutes, and the flow rate was recorded as millilitres per minute.

Stimulated whole saliva was recorded for 3 minutes in a similar manner, with moderate stimulation produced by having the patient chew on a piece of wax (abnormal less than or equal to 1.0 ml/min).<sup>(9)</sup>

### **3.4 Chart Review and Statistical Procedures**

The charts were reviewed by an optometry student who was trained by each of the examiners to interpret the entries on the charts. An Excel database was created to enter the data. When there were questions regarding the entries, such as unclear impressions or missing data, the rheumatologist was the final arbiter. A random sample of the charts was reviewed by the author to ensure the quality of the data entered.

A total of 420 charts were reviewed and 42 were excluded for: unclear SS diagnosis, absence of SS and Schirmer scores higher than 10mm, absence of SS and no

symptoms of dry eye. The study group included 231 pSS, 58 sSS and 89 KCS patients. The “control” dry eye patients were those with symptoms of dry eye for at least 3 months who also had a Schirmer score in the worst eye of 10 mm or less.

Factor analysis<sup>(3)</sup> was performed on the pSS database. Recursive partitioning<sup>(4)</sup> was used to create classification trees that demonstrated which characteristics best distinguished SS from KCS. Recursive partitioning uses a tree-structured set of questions about the descriptor variables to recursively divide the data into groups that are as homogeneous as possible, and the data is presented as nodes of a tree. To identify the best split for a specific node, the algorithm considers all possible binary splits for each descriptor variable and chooses the optimal one.<sup>(4)</sup> This splitting was performed to its completion and then pruned back to an appropriate size.<sup>(4)</sup>

For a categorical variable with only 2 levels there was only one split. If the categorical variable had more than 2 levels, any combination of levels was used to make the split. For numeric explanatory variables, the split was defined by values less than or more than a chosen value.

This data contained 231 pSS, 58 sSS and 89 KCS patients, and 90 variables as listed below. Several trees were created with various sets of variables including fully non-invasive selections.

### **3.4.1 List of variables (\* means non-invasive)**

The AECC criteria:

1. Criterion I: dry eye symptoms\*
2. Criterion II: dry mouth symptoms\*
3. Criterion III: dry eye signs (positive Schirmer or rose bengal)\*

4. Criteria IV: biopsy
5. Criterion V: salivary flow\*
6. Criterion VI: serum antibodies for Ro and/or La

Added details of the AECC criteria:

1. severity of dry eye symptoms (0-10)\*
2. how long eyes have been dry\*
3. severity of dry mouth symptoms (0-10)\*
4. how long mouth has been dry\*
5. rose bengal staining score (RB) of 4/9 or greater\*
6. RB value in worst eye 0-9\*
7. Schirmer failed, i.e., 5 or less\*
8. Schirmer value worst eye\*
9. biopsy focus score 0-4
10. Chisholm Mason biopsy score 3-4 is SS, i.e., at least 1 focus in 4 mm
11. unstimulated salivary flow\*
12. salivary flow score\*
13. Ro present
14. La present

Systemic autoimmune conditions:

1. mixed connective tissue (CT) disease present\*
2. CREST (calcinosis, Raynaud's, esophageal, sclerodactyly, telangiectasia) present\*
3. RA diagnosis\*

4. SLE diagnosis\*
5. PBC diagnosis\*

Other signs of AI disease:

1. Parotid swell\*
2. myalgia\*
3. arthralgia\*
4. fibromyalgia\*
5. lymphoma\*
6. X-ray positive\*

Other systemic diseases:

1. diabetes\*
2. hypothyroid\*

Blood work:

1. IgG
2. IgM
3. IgA
4. M spike
5. WBC
6. ANA
7. RF
8. ATA
9. Anti Mic

10. TSH
11. AMA
12. SMA

Other systemic symptoms:

1. dysphagia\*
2. dyspepsia\*
3. vaginal dryness\*
4. dysparunia\*
5. Raynaud's\*
6. dry skin\*
7. pruritis\*
8. rash\*
9. alopecia\*
10. photosensitive skin\*

Medications:

1. diuretics\*
2. depression\*
3. anticholinergics\*
4. anti-inflammatories\*

Other Eye Signs:

1. meibomian gland dysfunction\*
2. SLK\*



3. corneal stain of any kind\*

Rose bengal stain:

1. Worst eye (WE) RB temporal stain\*
2. WE corneal RB stain\*
3. WE nasal RB stain\*
4. each eye total of 3 areas as listed above\*

Fluorescein stain:

1. WE temporal cornea\*
2. WE nasal cornea\*
3. WE superior cornea\*
4. WE inferior cornea\*
5. WE central cornea\*
6. each cornea by 5 quadrants as listed above\*

Dental information:

1. missing teeth 0-33\*
2. filled teeth\*
3. cervical cavities\*
4. D score 0-33\*
5. DMF= dentate, missing filled\*
6. candidiasis\*

Demographics:

1. Age\*
2. Sex\*

\* designates a non-invasive test

### 3.5 Results

#### 3.5.1 Factor analysis of 231 pSS patients

The factor analysis produced 3 factors as follows:

1. Corneal staining with fluorescein and rose bengal
2. Conjunctival staining with rose bengal
3. Serum Ro and La antibodies

**Table 3.1: Factor analysis**

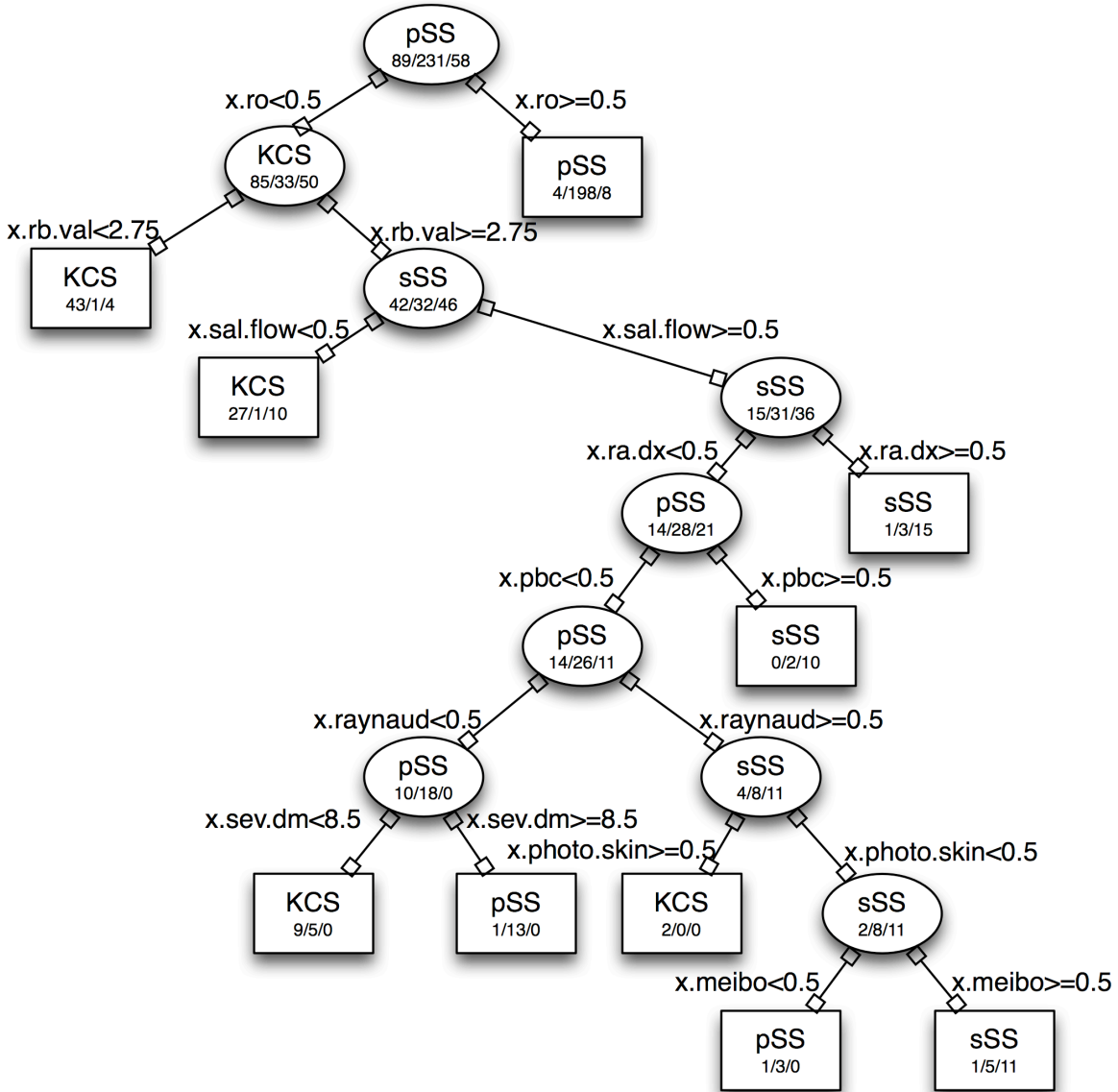
<b>Loading</b>	<b>Factor 1</b>	<b>Factor 2</b>	<b>Factor 3</b>
Worst eye temp corneal stain fl	0.876		
OD cornea temp fl	0.847		
OS cornea temp fl	0.813		
Worst eye cornea nasal fl	0.813		
OS nasal cornea fl	0.771		
OD nasal cornea fl	0.760		
Worst eye central cornea fl	0.584		
OS central cornea fl	0.583		
OD central cornea fl	0.558		

OD cornea anywhere fl	0.544		
Worst eye cornea RB	0.524		
Worst eye superior cornea fl	0.511		
OS superior cornea fl	0.501		
Worst eye RB temp		0.916	
Worst eye RB nasal		0.875	
OD RB temp		0.853	
RB value		0.829	
OS RB nasal		0.823	
OD RB nasal		0.808	
RB>3		0.795	
OS RB temporal		0.792	
Positive Ro			1.025
Positive Ro and/or La			1.024

Abbreviations: **fl** is fluorescein, **temp** is temporal, **RB** is rose bengal

### 3.5.2 Tree analysis results

**Figure 3.1: Tree #1: All Groups, All Non-Invasive Tests Include Salivary Flow, Plus Serum Ro and La Factors.**



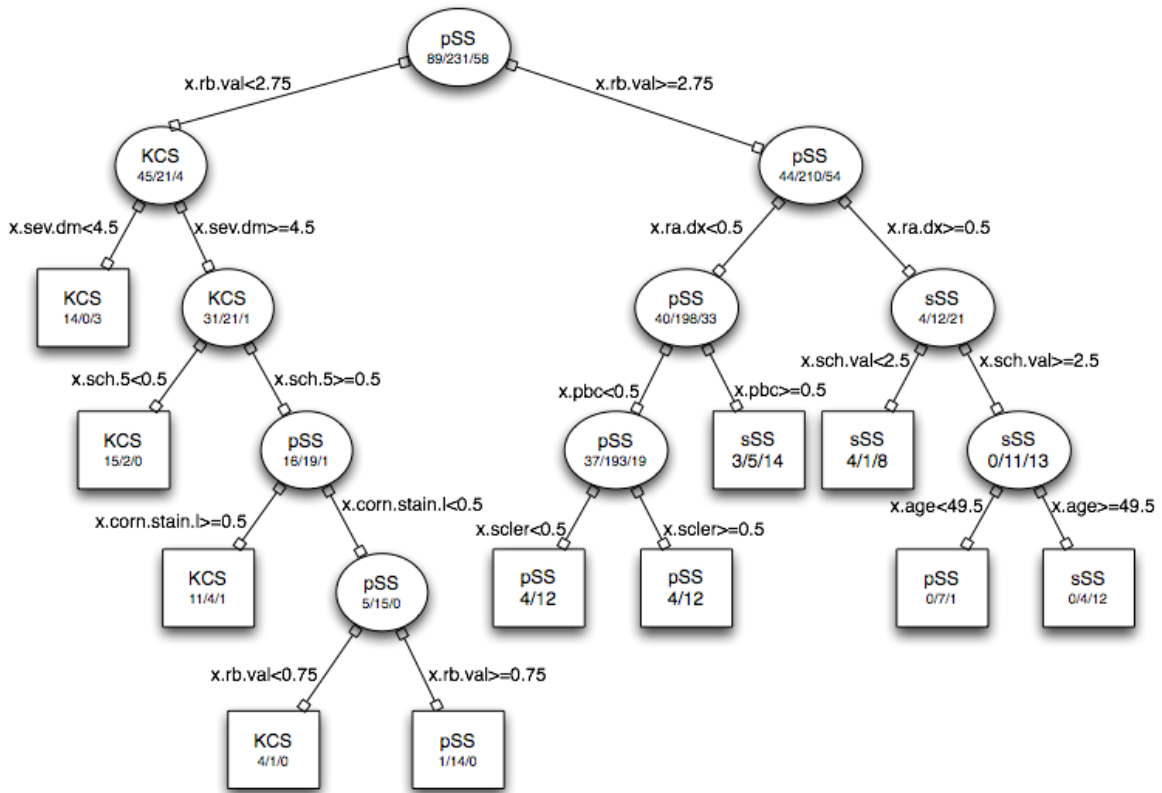
Legend for Tree #1: Numbers are listed as KCS/pSS/sSS, **x.ro** means anti-Ro protein in serum analysis, **x.rb.val** means total rose bengal score in worst eye, **x.sal.flow** means salivary flow test negative for SS, **x.ra.dx** means diagnosis of rheumatoid arthritis, **x.pbc** means diagnosis of primary biliary cirrhosis, **x.raynaud** means no diagnosis of Raynaud's phenomenon, **x.sev.dm** means the severity of dry mouth score, **x.photo.skin** means history of skin photosensitivity, **x.meibo** means observation of meibomian gland dysfunction.

**Table 3.2: Table for Tree #1: True vs Predicted with SS Grouped**

True	Predicted	Predicted
	KCS	SS
KCS	79	10
SS	21	268

As expected, the presence of anti-Ro immunoglobins in the serum best differentiates SS from KCS. The second most important variable in this tree is RB staining of the worst eye. KCS is best captured at a cut-off point of less than 2.75/9 in the worst eye. A positive salivary flow test then improves the diagnostic accuracy, leaving only 2 SS patients misdiagnosed as KCS. The specificity is not high, however, as 15 of the 89 KCS patients are misdiagnosed with SS at this point. The final sensitivity and specificity, after combining pSS and sSS, are as follows: sensitivity:  $268/289 = 92.73\%$  and specificity:  $79/89 = 88.75\%$ . This suggests that our tear tests, salivary flow test and a referral to family practice for simple Ro and La blood testing can lead to accurate referrals to rheumatology.

**Figure 3.2: Tree #2: 3 Groups: Non-Invasive Tests Only Without Salivary Flow**



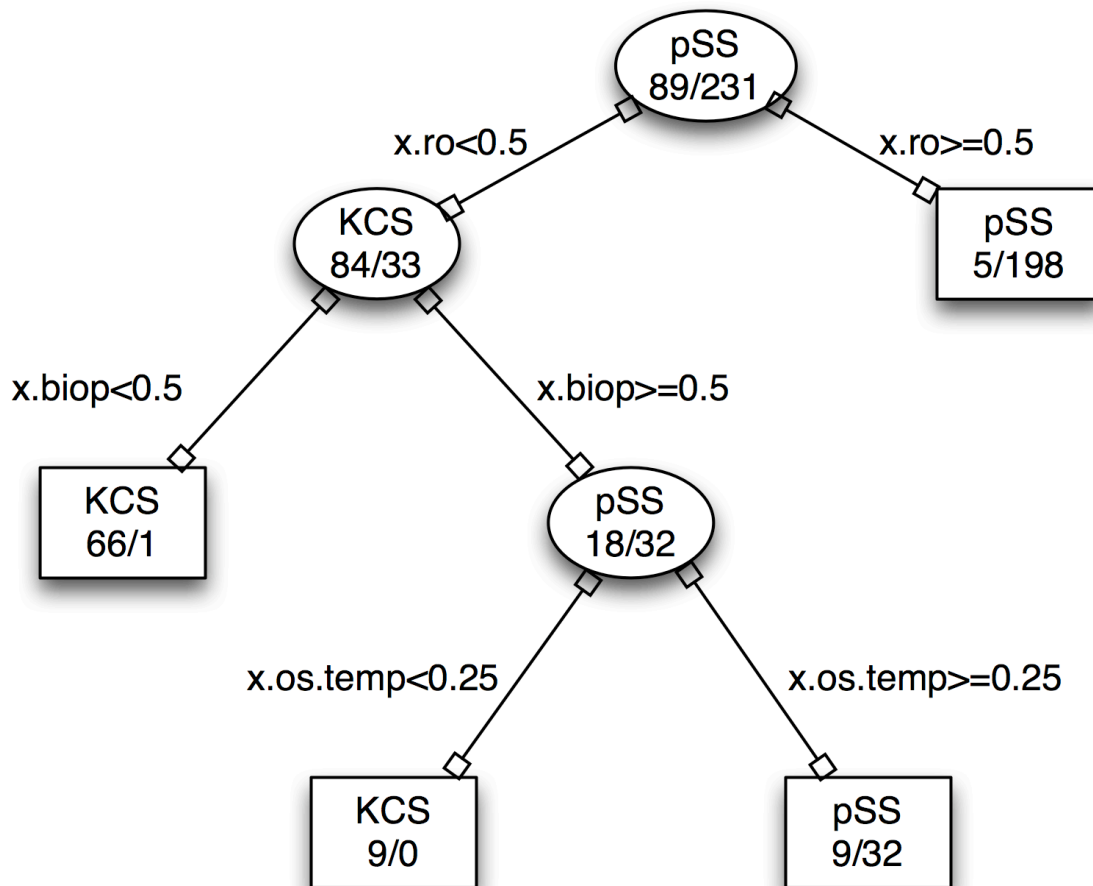
Legend for Tree #2: Numbers listed as KCS/pSS/sSS, **x.rb.val** means total rose bengal staining score in worst eye out of 9, **x.sev.dm** means patient rated severity of dry mouth out of 10, **x.sch.5** means Schirmer criterion for SS i.e.  $\leq 5$ mm in one eye, **x.corn.stain.I** means presence of corneal stain inferior quadrant, **x.ra.dx** means rheumatoid arthritis diagnosis, **x.sch.val** means actual Schirmer score, **x.pbc** means diagnosis of primary biliary cirrhosis, **x.age** means age of patient, **x.scler** means diagnosis of scleroderma.

**Table 3.3: Table for Tree #2: True vs Predicted, Non-Invasive Tests Only, SS Grouped**

True	Predicted	Predicted
	KCS	SS
KCS	44	11
SS	45	278

Of particular interest to eye care practitioners are those tests that we can do readily and non-invasively, without referral, that will help us to differentiate KCS and SS. If all 3 groups are used in the analysis, RB staining of 3 or more captures 210 of 231 pSS and 54 of 58 sSS. Unfortunately, the specificity of these simple tests is not high, and 44 of the 89 KCS patients are also included in this division. Here the sensitivity is  $278/289=96.19\%$  and the specificity is  $44/89=49.43\%$

**Figure 3.3: Tree #3: KCS and pSS Only, All Variables**



Legend for Tree #3: All numbers listed as KCS/pSS, **x.ro** means presence of anti-Ro immunoglobulin in serum, **x.biop** means biopsy score for SS, **x.os.temp** means temporal conjunctival staining with rose bengal.

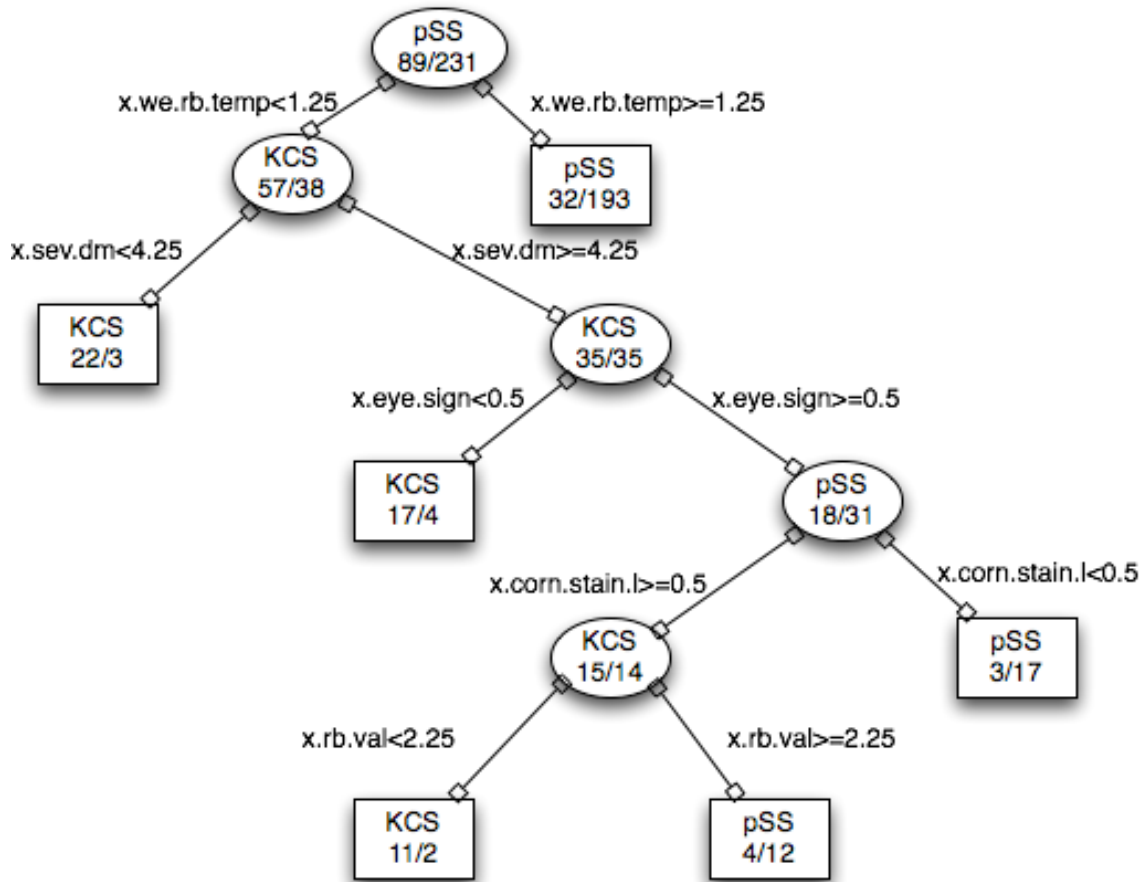
**Table 3.4: Table for Tree #3: True vs Predicted All Variables**

True	Predicted	Predicted
	KCS	pSS
KCS	75	14
pSS	1	230

Since the diagnosis of SS is both simplified and complicated by the presence of another autoimmune disease, pSS and KCS were examined alone. In this tree analysis, using all of the 83 variables, the sensitivity is very high at 99.57%. The one pSS patient that was not identified had a negative Ro and biopsy finding but was considered a pSS patient whose disease had burned out. The specificity was 84.26%. Of note is the fact that after the expected importance of Ro positivity and a positive biopsy score in the division of groups, the next most important variable was the presence of temporal conjunctival staining with rose bengal.



Figure 3.4: Tree #4: pSS and KCS Only: Non-Invasive Tests Without Salivary Flow



Legend for Tree #4: all numbers listed as KCS/pSS. **x.we.rb.temp** means temporal conjunctival staining with rose bengal of the worst eye, **x.sev.drm** means patient rating of severity of dry mouth out of 10, **x.eye.sign** means the presence of rose bengal staining greater than or equal 4 4/9 in the worst eye and/or a Schirmer score of less than or equal to 5mm in the worst eye, **x.corn.stain.I** means corneal staining with fluorescein in the inferior quadrant, **x.rb.val** means the value of rose bengal staining in worst eye out of 9.

**Table 3.5: Table for Tree #4: True vs Predicted**

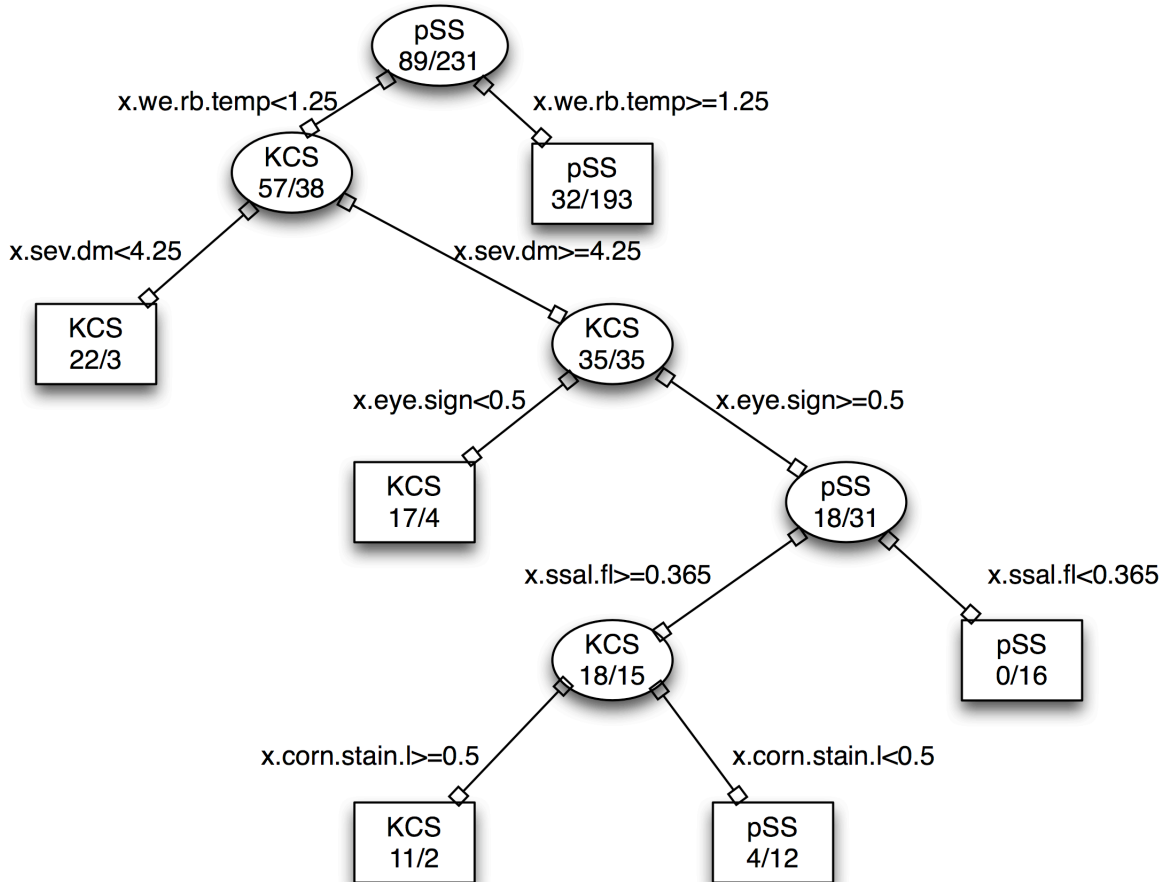
True	Predicted	Predicted
	KCS	pSS
KCS	50	39
pSS	10	221

In this tree analysis, using only non-invasive variables, it was shown that the most important variable in dividing pSS from KCS is RB staining of the temporal conjunctiva in the worst eye of greater than 1.25/3. One hundred and ninety-three (193) of 231 pSS patients are classified by this simple test. However, there are also 32 of 89 KCS patients falsely found by this variable. Of the 39 pSS patients that are not identified with the temporal RB stain variable, 35 of them are captured through a severity of dry mouth symptoms that is greater than 4.25/10. This second division misdiagnoses 35 KCS patients, 17 of whom can be differentiated from pSS by having either Schirmer scores greater than 5 in both eyes and/or rose bengal scores less than 4/9 in both eyes. Specificity is not high as 32 KCS patients are immediately grouped as pSS with the rose bengal score test, which in this tree analysis is a final node.

Of those 18 KCS patients who had worst eye temporal RB staining of less than 1.25 AND severity of dry mouth over 4.25 and either a positive RB score OR Schirmer scores less than or equal to 5 (n=18), 15 were differentiated by the presence of corneal fluorescein staining. It is noteworthy that 17 of the 31 SS patients did not have corneal staining. Finally, 11 of these KCS patients were differentiated as KCS by having a total RB score in the worst eye of less than 2.25/9. Using only temporal conjunctival staining, severity of dry mouth and either rose bengal total score of 4/9 or Schirmer less than or

equal to 5, only 10 of 231 SS patients are missed (sensitivity 95.7%). However, 39 of 89 KCS patients are misdiagnosed as pSS (specificity 56.18%).

**Figure 3.5: Tree #5: KCS and pSS Only: Non-Invasive Plus Salivary Flow**



Legend for Tree #5: All numbers listed as KCS/pSS, **x.we.rb.temp** means temporal conjunctival rose bengal staining in the worst eye, **x.sev.dm** means patient rating of severity of dry mouth out of 10, **x.eye.sign** means the presence of rose bengal staining greater than or equal to 4 4/9 in the worst eye and/or a Schirmer score of less than or equal to 5 mm in the worst eye, **x.ssal.fl** means amount of salivary flow per minute with stimulation, **x.corn.stain.l** means corneal staining with fluorescein in inferior quadrant.

**Table 3.6: Table for Tree #5: True vs Predicted Non-Invasive Plus Salivary**

True	Predicted	Predicted
	KCS	pSS
KCS	50	39
pSS	9	222

In observing the tree of pSS and KCS that adds salivary flow information, it is noteworthy that the sensitivity of the SS diagnosis remains high at 96.1% and the specificity remains low at 56.18%. Salivary flow testing did not improve the sensitivity to any great degree compared with strictly non-invasive testing. Of importance is the result that salivary flow information was secondary to rose bengal staining of the worst eye. Severity of dry mouth is the next most important variable in distinguishing KCS from pSS. Having low Schirmer scores and/or greater than or equal to 4/9 total RB score in the worst eye and having corneal staining are the other eye signs of importance in this tree analysis.

## **3.6 Discussion**

### **3.6.1 Results**

Factor analysis results suggest that corneal staining with fluorescein and conjunctival staining with rose bengal provide complementary yet separate information in diagnosing dry eye disease. This may help to explain the importance of conjunctival staining in capturing SS and in differentiating SS from KCS. The recursive partitioning analysis of this large cohort of dry eye patients demonstrates the importance of rose bengal staining of the conjunctiva in both identifying and differentiating SS. In addition,

factor analysis was used to provide a cohesive summary of the large amount of outcome variables.

### **3.6.2 The use of tree analysis**

Although the common approach to comparing such cohorts is analysis of variance,<sup>(10-13)</sup> this data was analyzed using recursive partitioning because of the nature of the database that had both missing data points and changes in procedures over time. For example, in our data, the stimulated salivary flow test was used until 2004, when a change to unstimulated salivary flow was undertaken. Also, as with most data associated with complex diseases, our data are influenced by the complex and unbalanced relationship among the clinical variables. Classification trees are suitable for such analysis because they deal well with nonlinear relationships, high-order interactions and missing values.<sup>(4, 14)</sup> They are also descriptive, in that the model represents the systemic structure of the data as simply as possible, as well as predictive, in that a model accurately predicts unobserved data.<sup>(4)</sup>

There is precedent for using recursive partitioning to evaluate the process of identification and differentiation of other rheumatic diseases<sup>(15, 16)</sup> and SS.<sup>(17)</sup> In 1996 Vitali et al. assessed the proposed preliminary criteria for the classification of SS by reviewing the clinical assessments of 278 cases (157 SS and 121 controls) collected from 16 centres in 10 countries. Each centre supplied 5 pSS, 5sSS and two sets of controls, 5 with a connective tissue disease but not SS and 5 who were patients with dry eyes and/or dry mouth symptoms who might be suspected of having SS.

They used recursive partitioning to analyze a subset of these patients, those with pSS and the combined subgroups of non-SS controls. This differs from our KCS

“controls,” as ours had both dry eye signs and symptoms. A significant difference in these two studies is also found in the choice of variables used in the tree analysis. They included only the 6 criteria of the proposed AECC. Their sensitivity was 95.6%, which is similar to ours, but their specificity was much higher at 90.9%.

Analysis of their tree shows that the first split was the presence of ocular or oral symptoms. The presence of oral or ocular dryness symptoms captured all but 1 of the pSS patients but did misclassify 48 of 77 controls. The absence of ocular signs (either Schirmer less than or equal to 5 and/or RB scores of 4/9 or greater, in the worst eye) captured 28 more of the controls. Histopathology results identified 26 of those 28 controls. Finally, salivary flow testing and the presence of serum anti-Ro or anti-La captured all but 7 of the 77 controls. In total only 3 of the 69 pSS patients were wrongly diagnosed.

The results reported here involved many more variables and had a completely different control group. However, it was shown that SS can be discovered in eye care offices with great sensitivity and can be differentiated from KCS to a moderate degree without invasive testing. In fact rose bengal staining of the temporal conjunctiva in the worst eye is a sensitive test for SS. Using rose bengal staining results combined with severity of dry mouth symptoms and other staining signs, we were able to capture all but 3 of the 231 pSS patients. This non-invasive evaluation demonstrates a sensitivity of 96.1%. The specificity of 56.2% would seem to be acceptable given the importance of the SS diagnosis. Throughout this analysis RB staining by location and severity proved to be an important differentiation of SS disease.

### **3.6.3 Limitations of this protocol**

There are some points that are noteworthy in this protocol. We used topical Alcaine (proparacaine hydrochloride 0.5%) prior to the instillation of 1% liquid rose bengal, and this may have influenced the results. The purpose of the anaesthetic was to reduce the distinct discomfort that accompanies the installation of rose bengal. Manning et al. in 1995<sup>(18)</sup> noted that rose bengal causes ocular irritation in patients with dry eye and remains in the damaged epithelium for hours after instillation. There is some belief that topical anaesthetics can contribute to staining.<sup>(19, 20)</sup> However, Forster found no such effect with tetracaine.<sup>(21)</sup> Although the anaesthetic was used on both groups, the effect may be different in SS patients and therefore may have caused some increase in staining. Nevertheless, even if there were something particular about the anaesthetic effect, in combination with the dye, it proved to be important diagnostically.

The selection of patients was biased in that the patients included in this study were referred by local ophthalmologists and rheumatologists. This bias would have affected any estimate of prevalence but did not limit the results of the tree analysis.

### **3.6.4 The importance of conjunctival staining**

The result that emphasizes the importance of conjunctival staining in the diagnosis of SS has not been described before. Ocular surface staining has been used by eye care practitioners for many years to observe the effects of dry eye and Sjogren's syndrome. However, in a recent Delphi panel only 65% of dry eye specialists reported using rose bengal regularly while 100% did use fluorescein.<sup>(22)</sup> Both the lack of availability of and the patient sensitivity to rose bengal may limit its use in clinical practice. The consistent use of 1% liquid rose bengal on a large cohort of dry eye patients

in this protocol may have allowed this variable to become evident. Also, since ocular surface staining is usually added in DE diagnosis, i.e., corneal and conjunctival staining are combined as in Criterion III for SS, clinicians may have missed the impact of conjunctival staining on its own in DE disease.

The type of dye that is used in clinical practice may also be an issue. Although the AECC criteria for SS diagnosis lists fluorescein and rose bengal in the description of the staining procedures,<sup>(2)</sup> rose bengal is considered the best dye for the observation of conjunctival staining, while fluorescein staining is very useful in observing the cornea.<sup>(23)</sup> Both Nichols et al.<sup>(24)</sup> and McCarty et al.<sup>(25)</sup> found a poor correlation between fluorescein and rose bengal conjunctival staining. Also, Yokoi and Kinoshita found that fluorescein was not a useful tool in severe dry eye cases.<sup>(26)</sup>

There is evidence that lissamine green can be used as a non-stinging substitute for rose bengal,<sup>(27)</sup> even though the staining mechanism is different. Although there is still much discussion about what characterizes a rose bengal stained conjunctival epithelial cell,<sup>(28)</sup> there is a belief that a compromise of the cell membrane mucous structure is essential.<sup>(19, 29), (30)</sup> In contrast, lissamine green is believed to stain cells that are dead or degenerated.<sup>(23)</sup> It is also known that normal healthy eyes display mild and transient conjunctival staining.<sup>(23)</sup>

Why temporal staining was such a strong variable makes for interesting speculation. Is this a circumscribed event that only occurs in certain forms of DE, or is it a measure of the severity of DE or the length of disease? Very little is known about the progress of corneal and conjunctival staining over time in dry eye disease. Some believe that conjunctival staining in dry eye begins in the nasal area and spreads to the temporal



area with progression of disease.<sup>(31, 32)</sup> Therefore temporal staining may be more prominent in SS because of the severity of dry eye in SS. The rationale here is that the flow of tears begins from the lacrimal gland to the temporal side of the globe and then towards the nasal globe through the menisci. With reduced tear clearance and the natural flow of the tears, the blink moves tears toward the nasal punctae, where there is a loss of water by evaporation and thus a concentration of tears, and inflammatory products at the nasal bulbar area that could be both hypersomotic and toxic.<sup>(33, 34)</sup>

Specific reference to temporal conjunctival fluorescein and rose bengal staining is rare in the literature but was noted to be one of the few dry eye observations that displayed moderate repeatability in a non-SS dry eye study by Nichols et al.<sup>(35)</sup> However, such staining was found in only 16% of their mild to moderate DE subjects.

Lissamine green has also been used to study SS dry eye. Begley et al., in 2003, compared lissamine green staining of the nasal and temporal bulbar conjunctiva in normals, non-SS dry eye and SS as diagnosed by the AECC.<sup>(36)</sup> They found that SS patients had significantly more nasal and temporal staining. Only 8.2% of non-SS dry eye subjects showed nasal staining versus 57.1% of the SS subjects, while temporal staining was present in 17% of the non-SS dry eye subjects compared with 48% of the SS patients. Corneal fluorescein staining was also higher in SS subjects in this study.

### **3.6.5 Conjunctival staining in other dry eye studies**

It seems reasonable that the degree of ocular surface staining would help to define SS-related dry eye, as SS is in part defined by its surface staining. The dry eye sign criterion includes staining of 4/9 or more in the worst eye as a partial criterion for SS.<sup>(2)</sup>

The importance of vital staining in SS and dry eye diagnosis is long-standing, and the degree of staining is considered to be greater in SS.

Others have used rose bengal stain to differentiate SS. Coll et al., in 1992, studied patients with various autoimmune diseases including those with diagnosed RA, scleroderma, PBC, other autoimmune liver disease, SLE and pSS as well as suspected SS patients.<sup>(37)</sup> The severity of rose bengal staining scores proved to have high specificity (98%), with low sensitivity (55%) for SS. They suggested that in patients with positive rose bengal staining and underactive salivary flow (based on salivary scintigraphy), lip biopsy was redundant for the diagnosis of SS. Our results show a much higher sensitivity (96.3%) for severity of rose bengal staining. Several differences are present in the two studies. First of all the AECC was not available to be used at the time of their study, and thus the diagnosis of the two SS populations differs. Of significance is the fact that they did not use anti-Ro and anti-La for diagnosis. Their diagnosis of SS required 2 of the following 3 results: KCS (Schirmer less than 5 AND grade B rose bengal staining OR grade A rose bengal staining), xerostomia (degree IV salivary gland biopsy AND degree III or IV salivary scintigraphy result), and autoimmune disease. Also they were comparing various autoimmune disease populations and not two groups of dry eye patients.

Tsubota et al., in 1994, compared subjects with SS, DE with serum autoantibodies but not SS and “simple” DE. Both fluorescein staining of the cornea and rose bengal staining of the conjunctiva were significantly worse in the SS group compared with the other groups, which did not differ.<sup>(38)</sup>

A recent paper by Versura et al.<sup>(39)</sup> also used the AECC in studying 62 SS patients and compared the clinical findings with 56 other autoimmune diseased patients and 59 sicca patients (diagnosis of symptoms of dry mouth and dry eye without serum positive scores for SS). Lissamine green scores showed high specificity (89%) and moderate sensitivity (63%) for SS. However, lissamine green stain was only applied to those patients who showed no fluorescein staining of the cornea, thus limiting the usefulness of this data in the comparison with our study.

### **3.6.6 Factors that influence conjunctival staining**

There may be many factors that cause increased amounts of staining and perhaps progression to the temporal conjunctiva in SS. The lacrimal gland influence would include reduced volume of secretion, an increase of inflammatory proteins being delivered from the gland to the ocular surface and reduced delivery of paracrine proteins. Thus the tear volume and composition would be altered and modified by lacrimal gland inflammation. The conjunctiva itself could contribute to this staining through changes in epithelial cells including altered mucin secretions and cytokine expression and from infiltration of lymphocytes and their products into the conjunctiva. Whether the increased SS staining is a result of a greater degree of these changes producing more staining in a final common pathway or a function of significant differences in the pathophysiology in SS versus DE is not known.

The SS patient's lacrimal gland is different from that of a non-SS dry eye patient. Although the pathology of the KCS lacrimal gland is poorly described, it does appear that significant histological differences exist, in that non-SS DE glands lack the specific inflammatory cells and pathognomonic foci associated with SS glands.<sup>(40, 41)</sup> This SS-

specific inflammation may have a greater effect on the reduced volume of tears and thus increase staining of the conjunctiva. Also anti-muscarinic proteins in the serum of many SS patients are believed to cause a greater reduction of lacrimal gland secretions in SS.<sup>(42)</sup>

Although there was a large range and no statistical significance (CI=95%) in the Schirmer scores of this population ([mean score +/-SD] pSS 2.86+/-3.12, sSS 2.72 +/-2.88, KCS 3.67+/-3.08, p=0.079), it is generally believed that SS patients have less tear flow. It is also suggested that the lack of reflex tearing that is attributed to the SS dry eye contributes to the increased staining in SS.<sup>(38, 43)</sup> Perhaps the occasional reflex tearing that occurs in non-SS DE is sufficient to provide paracrine proteins such as EGF to the conjunctival cells and thus reduce staining. It is believed that in most aqueous deficient dry eye diseases, and perhaps particularly SS, the reduced volume of tears increases tear osmolarity and subsequently increases the friction between lid and surface epithelia, both of which may cause greater staining.<sup>(44)</sup>

Several researchers have found a correlation between Schirmer scores and fluorescein and rose bengal staining in dry eye disease,<sup>(45-47)</sup> while Nichols et al. found fluorescein but not rose bengal stain to be correlated with Schirmer scores.<sup>(24)</sup> However, Tsubota et al. found no such correlation in DE or SS.<sup>(43)</sup>

### **3.6.7 Alterations in tear film composition**

Alterations in tear composition may contribute to staining and have been documented in DE and SS when compared with normal controls (see Tables 3.7 and 3.8). Reduced concentrations of lactoferrin (in DE<sup>(48, 49)</sup>; in SS<sup>(48, 50)</sup>), lysozyme (in DE<sup>(49)</sup>; in SS<sup>(51)</sup>) and EGF (in DE<sup>(48)</sup>; in SS<sup>(48, 52)</sup>) have been found in both DE and SS when compared with normal controls.

Also increased levels of IL-6 (in DE<sup>(53)</sup>; in SS<sup>(53, 54)</sup>), TNF- $\alpha$  (in DE and SS<sup>(53)</sup>) and MMP-3 in SS<sup>(55)</sup> have been discovered when compared with normal tears. Clearly the tear film composition is altered in many forms of DE disease.

Increased levels of other proteins have been noted in SS tears versus NDE controls. IL-1<sup>(55)</sup> and aquaporin-5<sup>(48)</sup> have been measured at elevated levels. We do not yet know how these proteins are represented in the tears of DE subjects.

Of greater importance to this discussion are the differences in tear composition between SS and DE and the effect that these may have upon ocular surface staining. Lactoferrin has consistently been found in lower concentrations in the tear film of SS versus DE subjects.<sup>(48, 50, 56)</sup> Of note is the fact that the Ohashi study<sup>(48)</sup> correlated reduced lactoferrin concentrations with rose bengal staining.

In one study, lipocalin was found to be reduced in SS tears compared with DE while no difference was found between DE and normal tears.<sup>(50)</sup> Lysozyme was found in reduced concentrations in SS versus DE in one study that demonstrated a trend but no statistical differences.<sup>(51)</sup> However, in a more recent study no statistically significant difference in tear lysozyme concentration was noted in SS, DE and normals.<sup>(50)</sup> One study found reduced concentrations of EGF in SS versus DE tears.<sup>(48)</sup>

In a comprehensive protein analysis of tears, large numbers of proteins were found in reduced concentration in SS versus DE, and this tear profile also correlated with RB staining.<sup>(57)</sup> In a more recent study, reduced total protein was also found in SS patients versus DE and not between DE and normals.<sup>(50)</sup>

The SS tear film differs in degree from DE in concentrations of certain proteins as well as in absolute values such as total tear protein. Both of these changes may contribute to the increased staining seen in SS dry eye.

**Table 3.7: Tear proteins in DE vs NDE (comparisons significant to p<0.05)**

<b>Tear component</b>	<b>DE vs Norm</b>	<b>Reference</b>	<b>Methods</b>	<b># Subjects</b>
Lactoferrin	Reduced	Ohashi, 2003 <sup>(48)</sup> Grus, 2005 <sup>(49)</sup>	ELISA Mass spec	71 DE, 16 NDE 88 DE, 71 NDE
Lysozyme	Reduced No difference	Grus, 2005 <sup>(49)</sup> Caffery, 2008 <sup>(50)</sup>	Mass spec Western blot	88 DE, 71 NDE 25 DE, 26 NDE
Lipocalin	No difference	Caffery, 2008 <sup>(50)</sup>	Western blot	25 DE, 26 NDE
EGF	Reduced	Ohashi, 2003 <sup>(48)</sup>	ELISA	71 DE, 16 NDE
IL-6	Increased	Yoon, 2007 <sup>(53)</sup>	ELISA	10 DE, 14 NDE
TNF- $\alpha$	Increased	Yoon, 2007 <sup>(53)</sup>	ELISA	10 DE, 14 NDE

Abbreviations: **DE** is dry eye, **NDE** is non-dry eye, **mass spec** is mass spectrometry, **EGF** is epidermal growth factor, **IL-6** is interleukin-6, **TNF- $\alpha$**  is tumor necrosis factor alpha.

**Table 3.8: Tear proteins in SS vs DE and NDE (comparisons significant to p<0.05)**

<b>Tear component</b>	<b>SS</b>	<b>Reference</b>	<b>Methods</b>	<b># Subjects</b>
Lactoferrin Protein	Reduced vs NDE	Ohashi, 2003 <sup>(48)</sup>	ELISA	23 SS, 16 NDE
	Reduced vs DE	Mackor, 1988 <sup>(56)</sup> Ohashi, 2003 <sup>(48)</sup>	Radial immunodiffusion ELISA	30 SS, 30 DE 23 SS, 71 DE
Lysozyme protein	Reduced vs NDE  No difference	Janssen, 1986 (trend only) <sup>(51)</sup>  Caffery, 2008 <sup>(50)</sup>	Radial immunodiffusion  Western blot	49 subjects range normal to severe DE  25SS, 26 NDE
Lipocalin protein	Reduced vs NDE and DE	Caffery, 2008 <sup>(50)</sup>	Western blot	25SS, 26 NDE
EGF protein	Reduced vs NDE	Pflugfelder, 1999 <sup>(52)</sup>	ELISA	10SS, 10 NDE
	Reduced vs DE	Ohashi, 2003 <sup>(48)</sup>	ELISA	23 SS, 71 DE
IL-1 protein	Increased vs NDE	Solomon, 2001 <sup>(55)</sup>	ELISA	9 SS, 17 NDE
IL-6 protein	Increased vs NDE	Tishler, 1998 <sup>(54)</sup>	ELISA	12 SS, 12 NDE
	Increased vs DE	Yoon, 2007 <sup>(53)</sup>	ELISA	8 SS, 10 DE
MMP-3 protein	Increased vs NDE	Soloman, 2001 <sup>(55)</sup>	ELISA	9 SS, 17 NDE
Aq-5 protein	Increased vs DE and NDE	Ohashi, 2003 <sup>(48)</sup>	ELISA	23 SS, 71 DE, 16 NDE

Total tear protein	Reduced vs DE and NDE	Caffery, 2008 <sup>(50)</sup>	Western blot	25SS, 26 NDE
Large #s of proteins	Reduced vs DE	Tomosugi, 2005 <sup>(57)</sup>	Mass spec	31 SS, 14 DE
TNF- $\alpha$ protein	Increased vs NDE	Yoon, 2007 <sup>(53)</sup>	ELISA	8 SS, 14 NDE
	No difference vs DE	Yoon, 2007 <sup>(53)</sup>	ELISA	8 SS, 71 DE
sIgA protein	No difference pSS vs sSS or NDE	Wehmeyer, 1991 <sup>(58)</sup>	ELISA	14pSS, 20 sSS, 23 NDE

Abbreviations: **DE** is dry eye, **NDE** is non-dry eye, **SS** is Sjogren's syndrome, **IL** is interleukin, **MMP** is matrix metalloproteinase, **Aq** is aquaporin, **mass spec** is mass spectrometry, **ELISA** is enzyme linked immunosorbent assay, **sIgA** is secretory immunoglobulin A

### 3.6.8 Alterations in mucins

Mucins of the ocular surface are altered in SS and other forms of DE, and it is suggested that these alterations also contribute to staining (see Tables 3.9 and 3.10). MUC5AC is a large gel-forming mucin that is secreted by the goblet cells of the conjunctiva.<sup>(59-61)</sup> MUC5AC plays an important role in the gel-forming aspects of the tear film that are protective of the underlying epithelium and may protect the epithelia from staining.<sup>(62)</sup> MUC19 is also a gel-forming mucin<sup>(63)</sup> expressed by corneal, conjunctival (including goblet cells) and lacrimal gland tissues.<sup>(64)</sup>



The non-goblet cell stratified epithelium of the conjunctiva produces transmembrane mucins<sup>(65)</sup> including MUC1,<sup>(66)</sup> MUC4<sup>(60)</sup> and MUC16.<sup>(67)</sup> These mucins have a membrane-spanning domain with a cytoplasmic tail and a long extra cellular domain that are believed to form the glycocalyx that covers the microplicae and microvilli of the epithelial cell surfaces. Transmembrane mucins have disadhesive properties<sup>(68)</sup> that protect epithelial cells by preventing cells from adhering to pathogens, debris and the lid epithelia<sup>(69)</sup> and may therefore protect the surface epithelia from staining.

The role of mucins in dry eye disease has mainly focused on MUC5AC, and it is generally assumed that the expression of MUC5AC is reduced in dry eyed subjects in the tear film<sup>(70, 71)</sup> and the conjunctiva.<sup>(70, 72, 73)</sup> Certainly reduced goblet cell density has been noted in SS<sup>(30, 74, 75)</sup> and DE<sup>(75-78)</sup> versus normals and in SS more than DE.<sup>(30)</sup> Little is written about the differences in gel-forming mucins between DE and SS.

Research has also been done to evaluate the role of membrane spanning mucins in dry eye disease.<sup>(30, 79, 80)</sup> Alterations in the distribution of conjunctival epithelial MUC16 have been noted in non-SS dry eye disease. Danjo et al. found that superficial temporal conjunctival epithelial cells did not bind the H185 antibody (MUC16) in non-SS dry eye as well as in normal subjects.<sup>(79)</sup> It is noteworthy that they found a significant correlation between staining scores of the temporal conjunctiva and this altered H185 (MUC16) binding pattern. Pflugfelder et al. also noted a correlation between membrane mucin absence and rose bengal staining.<sup>(30)</sup> To further the concept that mucin changes relate to staining, two studies used a human corneal-limbal epithelial cell line (HCLE) to demonstrate that MUC16 surface protein protects against rose bengal invasion.<sup>(80, 81)</sup>

However, a recent paper by Caffery et al.(82) has compared MUC16 expression in SS, DE and normals. MUC16 was found in higher concentration in the tears of SS subjects and MUC16 mRNA was found in increased concentration in the conjunctival epithelium of SS subjects (chapter 5). DE and normal control tears and conjunctival cells did not differ in their MUC16 expression in this study.

Data on other membrane bound mucins have suggested that the expression of mucosal epithelial membrane mucin (as detected by an uncharacterized antibody referred to as AMEM2) is reduced in SS and non-SS dry eyed subjects compared with controls and in SS versus DE.<sup>(30)</sup> Again these authors found a positive correlation between decreased transmembrane mucin (not identified) and higher rose bengal staining of the conjunctiva in aqueous deficient dry eye. However, other transmembrane mucins have not shown such differences in DE and controls. Argueso et al. failed to find differences in MUC1 or MUC4 gene expression between controls and SS subjects.<sup>(70)</sup>

Although little is known of the differences between DE and SS in the area of mucin expression, it appears that mucin changes of the ocular surface play a role in conjunctival staining and may, in future, help to explain the differences in SS and DE staining.

**Table 3.9: Mucin in DE vs NDE (comparisons significant to p<0.05)**

MUCIN	DE vs norms	Reference	Method	# Subjects
MUC5AC protein	Reduced	Nelson, 1986 <sup>(72)</sup>	Light microscopy	8 DE, 10 NDE
		Zhao, 2001 <sup>(71)</sup>	Immunochemical assay	31 DE, 19 NDE
MUC16 protein	Altered	Danjo, 1998 <sup>(79)</sup>	Immunohistochemistry EM	16 DE, 14 NDE

MUC1 gene	No change	Pisella, 2000 <sup>(78)</sup>	Flow cytometry	13 DE, 24 NDE
MUC1/A variant	Trend to lower vs NDE	Imbert, 2006 <sup>(83)</sup>	PCR	9 DE, 15 NDE
Goblet cell density	Reduced	Ralph, 1975 <sup>(76)</sup>	PAS stain	9 DE, 7 NDE
		Rivas, 1992 <sup>(77)</sup>	PAS stain	24 DE, 32 NDE
		Pisella, 2000 <sup>(78)</sup>	Immunofluorescence	5 DE, 4 NDE
		Kunert, 2002 <sup>(75)</sup>	PAS stain	15 DE, 11 NDE
Membrane mucin AMEM2	Reduced	Pflugfelder, 1997 <sup>(30)</sup>	Immunohistochemistry	9 DE, 10 NDE

Abbreviations: **MUC** is mucin, **DE** is dry eye, **NDE** is non-dry eye, **EM** is electron microscopy, **PCR** is polymerase chain reaction, **PAS** is periodic acid-Schiff stain

**Table 3.10: Mucin SS vs DE and NDE (comparisons significant to p<0.05)**

MUCIN	SS	Reference	Method	# Subjects
MUC5AC mRNA	Reduced vs NDE in conj	Argueso, 2002 <sup>(70)</sup>	PCR	11 SS, 17 NDE
MUC5AC protein	Reduced vs NDE in tears	Argueso, 2002 <sup>(70)</sup>	ELISA	11 SS, 17 NDE
MUC1 protein	Reduced vs NDE	Jones, 1998 <sup>(84)</sup>	Immunofl	8 SS, 8 NDE
MUC1 mRNA	No change vs NDE	Argueso, 2002 <sup>(70)</sup>	PCR	11 SS, 17 NDE
MUC4 mRNA	No change vs norms	Argueso, 2002 <sup>(70)</sup>	PCR	11 SS, 17 NDE
Glycocalyx structure	Absent vs NDE	Koufakis, 2006 <sup>(85)</sup>	TEM	8 SS, 7 DE

Goblet cell density	Reduced vs DE	Pflugfelder, 1990 <sup>(74)</sup>	PAS stain	19 SS, 17 DE
	Reduced vs NDE	Pflugfelder, 1997 <sup>(30)</sup>	PAS stain	11 SS, 10 NDE
		Kunert, 2002 <sup>(75)</sup>	PAS stain	12 SS, 11 NDE
Membrane mucin AMEM2	Reduced vs DE	Pflugfelder, 1997 <sup>(30)</sup>	Immunohist	11 SS, 10 NDE

Abbreviations: **SS** is Sjogren’s syndrome, **NDE** is non-dry eye, **MUC** is mucin, **mRNA** is messenger ribonucleic acid, **PCR** is polymerase chain reaction, **ELISA** is enzyme-linked immunosorbent assay, **Immunofl** is immunofluorescence, **PAS** is periodic acid-Schiff, **immunohist** is immunohistochemistry.

### 3.6.9 Alterations in inflammation

Inflammatory markers have also been studied in dry eye disease (see Tables 3.11 and 3.12). The conjunctiva itself has been implicated as an immunological target in SS by Pflugfelder et al.,<sup>(74)</sup> and the number of lymphocytes in conjunctival biopsies was measured as higher in SS patients for many years.<sup>(74, 86)</sup> However, more recently Stern et al.<sup>(87)</sup> and Gao et al.<sup>(88)</sup> found similar numbers and types of invasive lymphocytes in the conjunctivas of KCS and SS dry eye subjects.

The Stern et al. paper of 2002 is noteworthy, as the implication is that all aqueous deficient dry eye is the same and thus staining patterns in SS would be a result solely of the degree of dryness.<sup>(87)</sup> These authors did not use the AECC criterion for SS, and although the methods of diagnosis were not well described in the paper, it would appear that many KCS subjects might be included in their SS categorization. Regardless, this study demonstrated that large numbers of CD4+ T cells and markers of immune

inflammation of these T cells and the conjunctival epithelial cells were found in many KCS patients. Why these were not found on previous studies is not known.

Significant changes in the inflammatory nature of the conjunctiva have been identified in SS patients in the past as conjunctival cells increase their expression of inflammatory markers and cytokines.<sup>(84)</sup> For example, the immuno-inflammatory cell surface marker class II MHC antigen (HLA-DR) is expressed at higher levels in both SS<sup>(87, 89-91)</sup> and DE<sup>(31, 78, 92)</sup> subjects versus normals and in SS more than DE.<sup>(89)</sup> Also, intercellular adhesion molecule-1 (ICAM-1) is expressed at elevated levels in both SS<sup>(87, 91)</sup> and DE<sup>(78)</sup> versus normals. In contrast the Stern et al. paper does suggest that HLA-DR and I-CAM are expressed at equal levels by SS and DE subjects.<sup>(87)</sup>

The inflammatory cytokine interleukin-6 (IL-6) is expressed by conjunctival epithelial cells at elevated levels in SS versus normals.<sup>(52, 84, 93)</sup> No data exist on its presence in DE. Also, mRNA encoding TNF- $\alpha$ , IL-1 $\alpha$  and  $\beta$ , IL-6, IL-8, IL-10 and TGF- $\beta$ 1 is expressed at elevated levels in the conjunctiva of SS patients compared with normal controls.<sup>(52, 84)</sup>

Altered gene expression of keratinization proteins has been studied in SS patients in relationship to normals.<sup>(90)</sup> Keratin-6 and keratin-16, small protein rich protein 2A and kallikrein 7 were all upregulated in SS and suggest a keratinization pattern. Histological examination of superficial epithelial cells in various forms of DE disease demonstrated more squamous metaplasia in SS.<sup>(38)</sup>

**Table 3.11: Inflammatory markers in DE vs NDE (comparisons significant to p<0.05)**

Marker	DE vs NDE	Reference	Methods	# Subjects
HLA-DR protein	Increased	Pisella, 2000 <sup>(78)</sup>	Flow cytometry	13 DE, 12 NDE
		Brignole, 2000 <sup>(92)</sup>	Flow cytometry	99 DE, 50 NDE
		Rolando, 2005 <sup>(31)</sup>	Immunohistoch	32 DE, 16 NDE
ICAM-1 protein	Increased	Pisella, 2000 <sup>(78)</sup>	Flow cytometry	13 DE, 12 NDE

Abbreviations: **DE** is dry eye, **NDE** is non-dry eye, **HLA** is human leukocyte antigen, **immunohistoch** is immunohistochemistry, **ICAM** is intercellular adhesion molecule.

**Table 3.12: Inflammatory markers in SS vs DE and NDE (comparisons significant to p<0.05)**

Marker	SS	Reference	Methods	# Subjects
HLA-DR protein	Increased vs DE	Tsubota, 1999 <sup>(89, 94)</sup>	Flow cytometry	10 SS, 17 DE
	Increased vs NDE	Jones, 1994 <sup>(93)</sup>	Immunofluorescence	11 SS, 8 NDE
		Brignole, 2000 <sup>(92)</sup>	Flow cytometry	70 SS, 50 NDE
	Same as DE	Baudoin, 2002 <sup>(95)</sup>	Flow cytometry	68 SS, 101 DE
		Stern, 2002 <sup>(87)</sup>	Immunohistochemistry	15 SS, 15 DE
HLA-DR mRNA	Increased vs DE	Kawasaki, 2003 <sup>(90)</sup>	PCR	26SS, 30 NDE
ICAM-1 Protein	Increased vs NDE	Jones, 1994 <sup>(93)</sup>	Immunofluorescence	11 SS, 8 NDE
	Same as DE	Stern, 2002 <sup>(87)</sup>	Immunohistochemistry	15 SS, 15 DE

ICAM-1 mRNA	Increased vs NDE	Jones, 1994 <sup>(93)</sup>	PCR	10 SS, 11 NDE
		Gao, 2004 <sup>(88)</sup>	In situ hybridization	2 SS, 1 NDE
Lymphocytic infiltrate	Increased vs DE and NDE	Hikichi, 1993 <sup>(86)</sup>	Giemsa stain	40 SS, 11 DE, 10 NDE
		Pflugfelder, 1990 <sup>(74)</sup>	PAS stain	19 SS, 17 DE, 9 NDE
	Same as DE	Stern, 2002 <sup>(87)</sup>	Immunohistochemistry	15 SS, 15 DE
	Gao, 2004 <sup>(88)</sup>	Immunohistochemistry	2 SS, 8 DE	
IL-6 mRNA	Increased vs NDE	Jones, 1994 <sup>(93)</sup>	PCR	10 SS, 11 NDE
		Jones, 1998 <sup>(84)</sup>	PCR	3 SS, 3 NDE
		Pflugfelder, 1999 <sup>(52)</sup>	PCR	10 SS, 10 NDE
TNF- $\alpha$ mRNA	Increased vs NDE	Jones, 1998 <sup>(84)</sup>	PCR	3 SS, 3 NDE
		Pflugfelder, 1999 <sup>(52)</sup>	PCR	10SS, 10 NDE
TGF- $\beta$ 1 mRNA	Increased vs NDE	Jones, 1998 <sup>(84)</sup>	PCR	3 SS, 3 NDE
		Pflugfelder, 1999 <sup>(52)</sup>	PCR	10 SS, 10 NDE

Abbreviations: **SS** is Sjogren's syndrome, **DE** is dry eye, **NDE** is non-dry eye, **HLA** is human leukocyte antigen, **mRNA** is messenger ribonucleic acid, **ICAM** is intercellular adhesion molecule, **PCR** is polymerase chain reaction, **PAS** is periodic acid-Schiff stain, **IL** is interleukin, **TNF** is tumor necrosis factor, **TGF** is transforming growth factor.

In summary, both DE and SS demonstrate alterations in lymphocytic infiltration to the conjunctiva, as well as increases in inflammatory markers such as HLA-DR and ICAM-1. Historically, these levels are greater in SS. However, the most recent paper by

Stern et al. suggests that KCS and SS DE are not different in their infiltration and immune activation. The only inflammatory cytokine that has been noted as increased in the conjunctiva of SS versus DE patients is TNF- $\alpha$ . Whether these differences influence conjunctival staining patterns is not known.

The meibomian glands of SS patients have been shown to be more severely impaired and inflamed than those of DE patients and therefore are believed to enhance ocular surface staining seen in this disease, secondary to meibomian inflammation,<sup>(96, 97)</sup> or from a change in surface tension.<sup>(97)</sup>

### **3.7 Conclusions**

The algorithm created through tree analysis demonstrates that eye care practitioners can contribute to the diagnosis and differentiation of Sjogren's syndrome through the use of rose bengal staining. SS should be entertained as a diagnosis particularly in post menopausal female patient presenting with dry eye symptoms and/or another autoimmune disease. The fact that SS patients have more conjunctival staining may be explained by either the quantity or quality of changes in the lacrimal glands, surface epithelia and tear film. The most severe form of dry eye is SS, and it may be that it simply lies at the far end of a spectrum of dry eye disease. It is also likely that the type of inflammation of the lacrimal gland and the ocular surface is different in SS.

As new information becomes available, the challenges of the bulbar conjunctiva in SS become clearer. It may be that the ocular SS pathology begins with mechanical abrasions that are a part of an increased friction between lid and epithelium secondary to a reduced tear film. Cells fail to produce enough mucin and eventually lose their wet phenotype. The number of goblet cells being produced or surviving is reduced, which



results in an even poorer tear film as a result of inadequate gel-forming mucins to stabilize the tear film. Distressed surface cells begin to respond with expression of inflammatory cytokines that change the tear film and direct lymphocytes to the area. The elevated cytokine levels, combined with the reduced lacrimal secretion elements such as EGF and retinol, create an environment where terminal differentiation of the ocular surface epithelium is further impaired. These changes are best seen by the eye care practitioner through the use of rose bengal stain.

In conclusion, the identification of Sjogren's syndrome and its differentiation from other forms of dry eye disease can be improved in the eye care office by grading rose bengal staining of the conjunctiva, particularly the temporal area. Adding a dry mouth questionnaire and where necessary referring the patient for simple Ro and La blood analysis will improve both sensitivity and specificity of referrals to rheumatology.

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## 4 Tear Proteins in Sjogren's Syndrome

This chapter is the basis of a paper published in 2008: Caffery B, Joyce E, Boone A, et al. Tear lipocalin and lysozyme in Sjogren and non-Sjogren dry eye. *Optom Vis Sci* 2008;85:661-7.

### 4.1 Overview

**Purpose:** To investigate the relationship between tear flow and tear lipocalin, lysozyme and total protein concentrations in non-dry-eyed subjects (NDE) and in two distinct groups of dry-eyed subjects: those with keratoconjunctivitis sicca (KCS) and those with primary Sjogren's syndrome.

**Methods:** Seventy-six (76) subjects were recruited for this study: 25 primary Sjogren's subjects (SS); 25 non-Sjogren's KCS subjects (KCS) and 26 healthy non-dry-eyed individuals (NDE). Tear flow was measured by the Schirmer I test without anesthesia. Tears were collected using an eye wash. Total tear protein was quantified using the DC Protein Assay Kit<sup>®</sup>. Tear lipocalin and lysozyme were quantified via Western blotting performed on a Phast<sup>®</sup> System.

**Results:** By definition, the SS and KCS groups both had significantly lower mean Schirmer scores ( $5.12 \pm 5.96$  mm and  $7.84 \pm 7.35$  mm) compared with the NDE group ( $23.83 \pm 7.85$  mm). There was no difference in mean Schirmer scores between SS and KSC groups ( $p=0.19$ ). The tear film of the SS group was characterized by significantly reduced ( $p<0.0001$ ) total protein and lipocalin concentrations compared with both KCS

and NDE groups. No difference between the KCS and NDE groups was found in total protein ( $p=0.92$ ) or lipocalin ( $p=0.19$ ) concentration. In contrast, the concentration of tear film lysozyme was found to be statistically similar in all three groups examined. No statistically significant correlation was found in any group between mean Schirmer values compared with total protein, lipocalin or lysozyme concentration.

**Conclusion:** These results demonstrate a biochemical distinction of the Sjogren's group compared with both KCS and control groups, in that both tear lipocalin and total tear protein were significantly reduced. Although correlations were not found between protein measurements and tear flow, a combination of tests including Schirmer I and quantitation of tear film biomarkers may facilitate differential dry eye diagnosis.

## 4.2 Introduction

Dry eye is defined as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance and tear film instability with potential damage to the ocular surface.<sup>1</sup> The 2007 Report of the International Dry Eye Workshop (DEWS) categorized dry eye into two main etiological branches: aqueous deficient (AD) and evaporative.<sup>1</sup> The AD dry eye branch is divided into two subgroups: Sjogren's syndrome (SS) dry eye and non-Sjogren's dry eye. As the classification implies, both AD subgroups exhibit reduced secretions from the lacrimal gland. As a result, clinical diagnosis is based in part on measurements of tear secretion or volume. A Schirmer I test is often used to establish the secretory capability of the lacrimal gland. Non-Sjogren's AD dry eye is defined differently by various authors, but a tear flow of less than 10 mm in 5 minutes, combined with symptoms of irritation, is often sufficient for the diagnosis of AD dry eye or keratoconjunctivitis sicca (KCS). The Schirmer score criterion<sup>2</sup> for SS is  $\leq 5$  mm in 5 minutes.

Evaluation of tear biochemistry from KCS patients suggests that reduced tear flow may be accompanied by various alterations in protein concentrations.<sup>3-8</sup> The mechanisms underlying such changes are currently unclear. The healthy lacrimal gland secretes a complex fluid that contains proteins, nutrients, hormones, growth factors and immunoglobins in an isotonic electrolyte solution. Regulated proteins are synthesized in the endoplasmic reticulum of the acinar cells, modified in the Golgi apparatus and stored in the secretory granules that fill the apical region of the cell.<sup>9-10</sup> Upon stimulation, the secretory granules fuse with the apical membrane and proteins such as lysozyme,

lipocalin and lactoferrin are released into the lumen and incorporated into bulk aqueous flow.<sup>9-10</sup>

Lacrimal gland secretory insufficiency in SS is, in part, a function of lymphocytic infiltration.<sup>11</sup> This invasive change was, at one time, believed to be the sole cause of reduced secretion. It is now understood that multiple mechanisms, including neurological influences, participate in SS acinar cell malfunction.<sup>11-14</sup> Although the pathology of the KCS lacrimal gland is poorly defined in comparison with the SS lacrimal gland, it does appear that significant histological differences exist, in that KCS glands lack the inflammatory cells and pathognomonic foci associated with SS glands.<sup>15-16</sup> Thus, despite that both SS and KCS clinically present with abnormally low tear flow, the lacrimal gland pathophysiology underlying the secretory malfunction is presumed to be different.

In support of this assumption, numerous studies have differentiated SS from KCS and control populations based on variations in tear film protein expression,<sup>3-7,17-18</sup> leading to the suggestion that biomarker profiling may offer significant utility in dry eye diagnosis.<sup>2,6,7,17</sup> Although combining multiple biomarkers may provide the most accurate ability to differentiate between subgroups of dry-eyed patients,<sup>2,7,17</sup> at present such analysis may be too complicated for routine clinical application. In place of detailed biochemical profiling, numerous studies have suggested that quantitation of a single biomarker such as lysozyme,<sup>20-22</sup> lipocalin<sup>4</sup> or lactoferrin<sup>3,5,17,22,23</sup> could serve as a supplemental diagnostic parameter, alongside traditional assessments of the ocular surface.

The purpose of this study was to investigate the relationships between tear secretion and levels of tear film lipocalin, lysozyme and total protein concentrations in

two distinct groups of dry-eyed subjects: those with keratoconjunctivitis sicca and those with primary Sjogren's syndrome, as well as a non-dry-eyed control group. Through such an evaluation, we sought to gain insight into potential clinical–biochemical relationships and to gauge the utility of these protein biomarkers for the diagnosis of dry eye and the differentiation of dry-eyed subgroups.

### **4.3 Materials and Methods**

#### **4.3.1 Study design**

Prior to the start of this study, ethics approval was attained from the Offices of Research at the University of Waterloo and the University of Toronto, and all procedures adhered to the Declaration of Helsinki. A total of 76 subjects – 26 non-dry-eyed controls (NDE), 25 Sjogren's subjects (SS) and 25 non-Sjogren's keratoconjunctivitis subjects (KCS) – were enrolled in this study. All subjects were free from allergy or other ocular surface diseases, and all were on maximum therapy for blepharitis, if that condition had been previously diagnosed. Thus, where appropriate, they were using lid scrubs and hot soaks but were not using topical antibiotics or topical anti-inflammatories. All participants underwent a clinical evaluation visit to determine entry eligibility, prior to a second visit in which tear samples were collected.

All SS participants had been diagnosed with primary SS at the Sjogren's Syndrome Clinic of the University Health Network in Toronto, using the American-European consensus criteria of 2002.<sup>2</sup> Recruitment of these patients was done through telephone calls from the database of that clinic. No further preliminary screening was performed on this group.

The KCS and NDE subjects were recruited through the SS clinic and a private practice. Participants first answered the question, “If you have dry eyes, have they been dry for at least 3 months?” Participants who answered yes were asked to rate their dryness on a visual analogue scale used routinely in the SS clinic. The horizontal line of the scale was marked from 0 to 10. At the 0 point the phrase “not dry at all” was written and at the 10 point “as dry as the desert” was written. If the subject rated his or her dryness as greater than or equal to 6 out of 10 and the Schirmer 1 test score was less than or equal to 10 mm in 5 minutes in at least one eye, they were described as KCS. Non-dry eye subjects (NDE) were enrolled if they stated that they did not have dry eyes and had Schirmer I scores of greater than 10 mm. All of the recruitment testing of KCS and NDE subjects was done within two months of the collection of tears. The Schirmer I test was performed using prepackaged, sterile paper strips (Schirmer Tear Test Strips<sup>®</sup>, Alcon, Fort Worth, TX), without anesthesia. The rounded bulb end of the strip was folded at the notch and then inserted into the lower fornix, one third of the distance from the lateral canthus of the lower lid. Once both strips were in place, the subject was asked to close his or her eyes. After 5 minutes, the strips were removed and the wet portion measured in millimetres.

#### **4.3.2 Analytical techniques**

**Reagents and Materials:** All PhastSystem<sup>™</sup> pre-cast gels, buffer strips, well combs, filter paper and ECL-Plus<sup>™</sup> kits were purchased from GE Healthcare (Baie d'Urfe, QC, Canada). Immuno-Blot<sup>®</sup> PVDF (polyvinylidene difluoride) membrane and DC Protein Assay Kit<sup>®</sup> were purchased from BioRad Laboratories (Mississauga, ON, Canada). Polyclonal rabbit anti-human lysozyme from Nordic Immunological Laboratories

(Tilburg, the Netherlands), monoclonal mouse anti-human lipocalin from R&D Systems (Minneapolis, MN, USA), goat anti-mouse IgG-HRP from Jackson ImmunoResearch (West Grove, PA, USA), and human lysozyme (neutrophil) from Calbiochem (La Jolla, CA, USA) were purchased from the distributor Cedarlane Laboratories (Hornby, ON, Canada). Goat anti-rabbit IgG-HRP and all other analytical grade reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

### **4.3.3 Tear wash collection**

Tears were collected using a wash method as described previously.<sup>24</sup> The right upper lid of each subject was held open and 60  $\mu$ l of sterile, physiological saline (Minims<sup>®</sup>, Chauvin Pharmaceuticals Limited, Romford, Essex, UK) was instilled onto the superior bulbar region of the unanaesthetized ocular surface using a sterile micropipette (VWR Scientific, Mississauga, ON, Canada). Without blinking, each subject was asked to rotate the eye to spread the saline over the ocular surface. Tear wash was then collected from the inferior fornix using the sterile micropipette. The same procedures were then repeated in the left eye. Washes were pooled together and stored at -80°C until use. 5  $\mu$ L of eye wash diluted in 5  $\mu$ L of distilled water was used to calculate total tear film protein using the DC Protein Assay Kit<sup>®</sup>, following the manufacturer's instructions. Duplicate samples were analyzed and data were read at 750 nm on a Multiskan Microplate spectrophotometer (Thermo Labsystems, Franklin, MA, USA).

### **4.3.4 Electrophoresis and immunoblotting**

For analysis of lipocalin, tear samples were diluted to 15 ng total protein/ $\mu$ L with modified Laemmli's buffer (50 mM Tris-HCl, pH 7.4, 2.5% sodium dodecyl sulfate



[SDS] w/v, 2.5% glycerol, 5 mM dithiothreitol [DTT], 0.01% bromophenol blue). For lysozyme analysis, tear samples were diluted with gel loading buffer (60 mM Tris [pH 6.8], 2% glycerol, 2% SDS, 0.01% bromophenol blue) to a final concentration of 15 ng total protein/ $\mu$ L. To facilitate quantitation of tear samples, standard curves were run on each gel. For lipocalin, this was a titration (5 to 30 ng/ $\mu$ L total protein) of pooled human tears collected from non dry-eyed volunteers and for lysozyme, a titration (1 to 10 ng/ $\mu$ L) of human neutrophil lysozyme. Once prepared, samples and standards were subjected to SDS-PAGE followed by Western blotting to PVDF membranes using the PhastSystem™ (GE Healthcare, Baie d'Urfe, QC, Canada). Lipocalin was identified through incubation with a mouse anti-human lipocalin monoclonal antibody (1:20 000) diluted in TBS + 0.05% Tween 20 (TBS-T) for 2 hours followed by a 1 hour incubation with goat anti-mouse secondary antibody (1:10 000) diluted in TBS-T. Lysozyme was identified using a rabbit anti-human lysozyme polyclonal antibody (1:1000) in TBS-T with 5% skim milk powder for 2 hours, followed by peroxidase conjugated goat anti-rabbit secondary antibody (1:20 000) in TBS-T with 5% skim milk powder for 1 hour. Immunoreactivity was visualized by incubating with ECL-Plus™ chemiluminescent substrate. Optical densities of the resulting bands were quantified from digitized images created with a Molecular® Dynamics Storm™ 840 Imager using ImageQuant™ 5.1 (GE Healthcare, Baie d'Urfe, QC, Canada). Regression analysis was performed from standard curve data to generate standardized values of tear film lipocalin and lysozyme. Lipocalin data are expressed as arbitrary units (AU) per  $\mu$ g total protein whereas lysozyme data are expressed as  $\mu$ g per  $\mu$ g total protein.

### 4.3.5 Data analysis

Statistical analyses were performed using Microsoft Excel™ XLfit® software. All data are reported as mean  $\pm$  standard deviation. Statistical differences between groups were analyzed by analysis of variance followed by post-hoc Dunnett's comparison of means and by Tukey's test. Significance was identified at  $p < 0.05$  ( $\alpha = 0.05$ ). Pearson's coefficient of correlations between tear flow and protein measurements was calculated in Excel™.

## 4.4 Results

### 4.4.1 Demographics and tear flow measurements

A total of 76 subjects were enrolled into this study. Demographics are displayed in Table 4.1. Mean age of the SS group was found to be statistically higher than the NDE group ( $p = 0.024$ ). Mean Schirmer I scores from both eyes collected without anesthesia for five minutes revealed a significantly reduced tear flow in both SS ( $5.12 \pm 5.96$  mm) and KCS subjects ( $7.84 \pm 7.35$  mm), relative to NDE ( $23.83 \pm 7.85$  mm),  $p < 0.0001$ . There was no difference in mean Schirmer I scores between the KCS and SS groups ( $p = 0.19$ ).

**Table 4.1: Summary of demographic information for study groups**

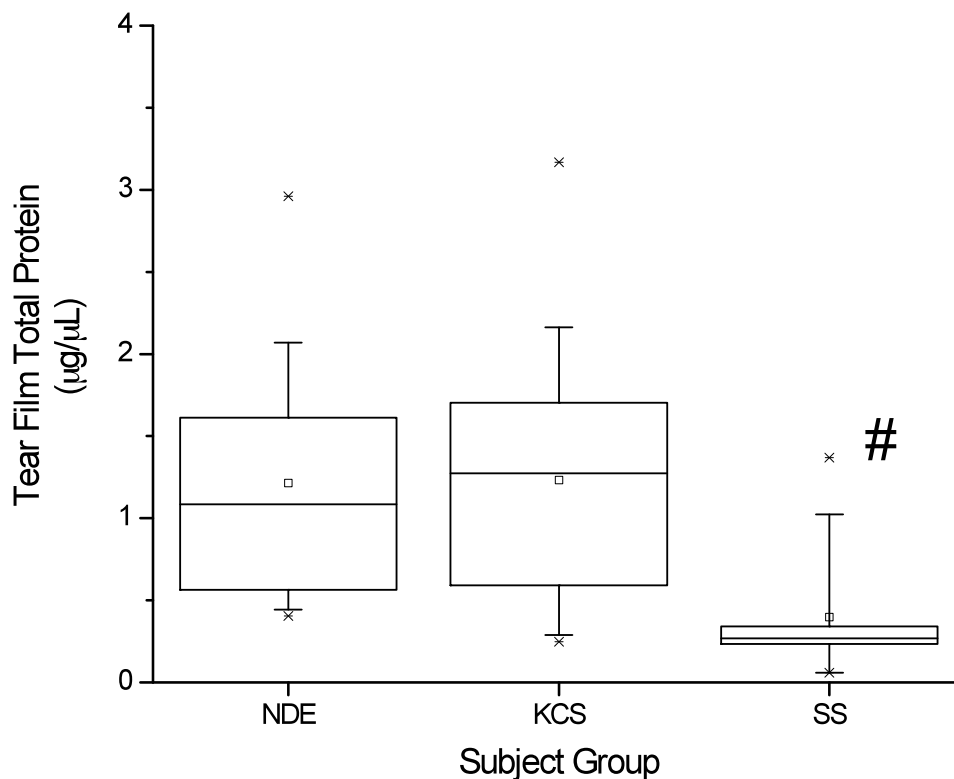
GROUP	MEAN AGE (YEARS)	NUMBER OF FEMALE SUBJECTS	NUMBER OF MALE SUBJECTS	TOTAL SUBJECTS
NDE CONTROL	$52.4 \pm 11.4$	24	2	26
KCS	$59.3 \pm 9.1$	21	4	25
SS	$60 \pm 11.8^*$	21	4	25

\* denotes significantly greater compared with control group ( $p = 0.024$ )

#### 4.4.2 Total tear protein concentration

Figure 4.1 displays mean tear total protein concentrations for the three groups. Tear total protein concentration was significantly lower in the SS group ( $0.4 \pm 0.32 \mu\text{g}/\mu\text{L}$ ) compared with the KCS group ( $1.23 \mu\text{g}/\mu\text{L}$ ;  $p < 0.0001$ ) and the NDE group ( $1.21 \mu\text{g}/\mu\text{L}$ ;  $p < 0.0001$ ). No difference in tear total protein concentration was found between the KCS and NDE groups ( $p = 0.92$ ). No correlation was found between tear film total protein concentration and tear flow measurements, as assessed by the Schirmer I test (Table 4.2).

**Figure 4.1: Box plots of total tear film protein concentrations**



Inner boxes represent mean, line within large box represents median, large boxes represent standard error, whiskers represent standard deviation and (\*) represents range of tear film protein concentration from non dry-eyed (NDE), KCS subjects (KCS) and Sjogren's subjects (SS). # denotes significantly lower relative to both NDE and KCS ( $p < 0.0001$ ).

**Table 4.2: Summary of correlation coefficients calculated for mean tear flow values compared with tear protein measurements**

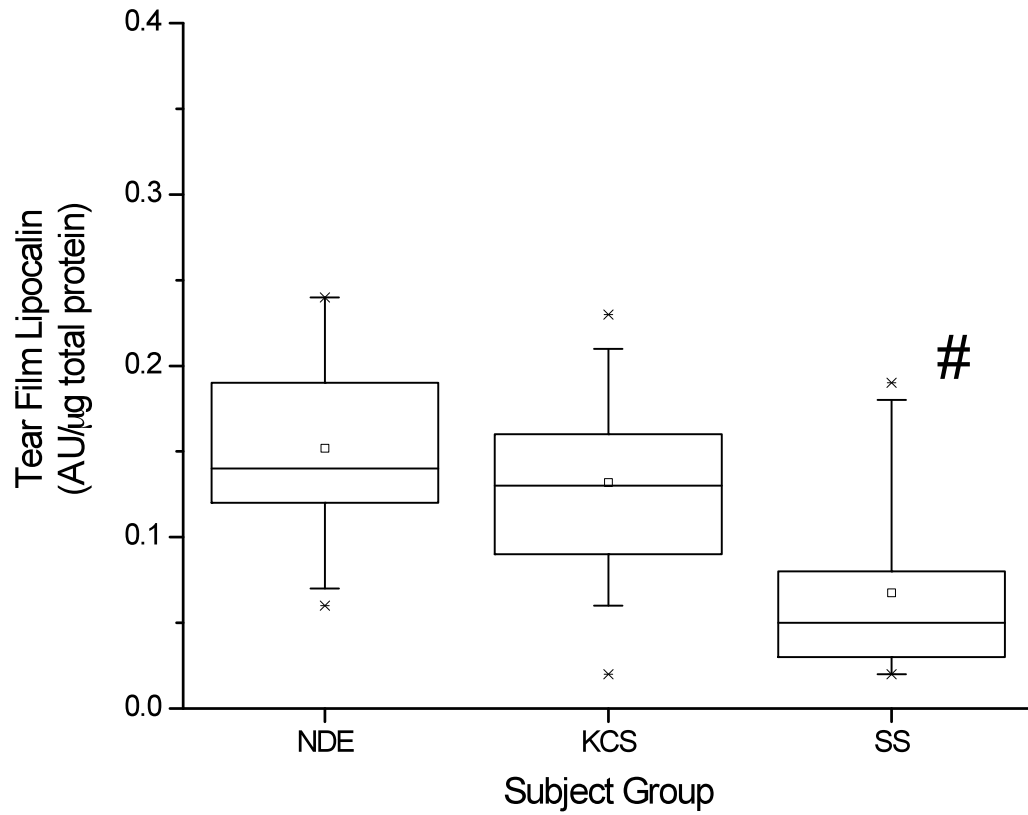
<b>GROUP</b>	<b>TOTAL PROTEIN CONCENTRATION</b>	<b>LIPOCALIN CONCENTRATION</b>	<b>LYSOZYME CONCENTRATION</b>
NDE CONTROL (N=26)	0.27	-0.02	0.49
KCS (N=25)	-0.04	0.49	0.29
SS (N=20)	0.02	0.29	-0.004

#### **4.4.3 Tear film lipocalin and lysozyme concentrations**

As shown in Figure 4.2, the SS group displayed a significantly reduced ( $p < 0.0001$ ) lipocalin concentration ( $0.067 \pm 0.05$  AU) compared with both KCS and NDE groups ( $0.13 \pm 0.05$  and  $0.15 \pm 0.05$  AU, respectively). No difference in tear film lipocalin was found between KCS and NDE groups ( $p = 0.19$ ). No correlation was found between tear lipocalin concentration and tear flow (Table 4.2).

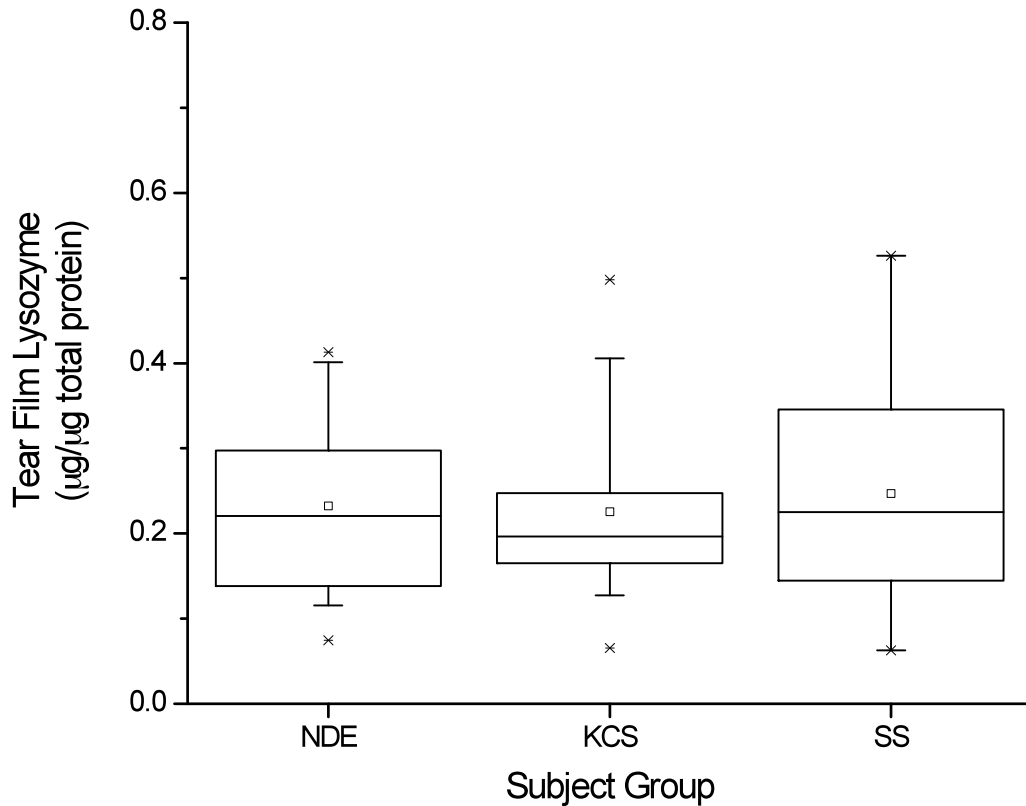
No difference ( $p = 0.80$ ) was found between any of the groups with respect to the concentration of tear film lysozyme, as summarized in Figure 4.3. No correlation was found between tear lysozyme concentration and tear flow (Table 4.2). Directly comparing the concentrations of lipocalin and lysozyme from individual samples also failed to identify statistically relevant correlations (NDE = 0.42, KCS = 0.07, Sjogrens = 0.11).

**Figure 4.2: Box plots of tear film lipocalin concentrations**



Inner boxes represent mean, line within large box represents median, large boxes represent standard error, whiskers represent standard deviation and (\*) represents range of tear film lipocalin concentration from non dry-eyed (NDE), KCS subjects (KCS) and Sjogrens subjects (SS). # denotes significantly lower relative to both NDE and KCS ( $p < 0.0001$ ).

**Figure 4.3: Box plots of tear film lysozyme concentration**



Inner boxes represent mean, line within large box represents median, large boxes represent standard error, whiskers represent standard deviation and (\*) represents range of tear film lysozyme concentration from non dry-eyed (NDE), KCS subjects (KCS) and Sjogren's subjects (SS).

## 4.5 Discussion

Our results clearly demonstrate distinct tear film chemistries, not only between dry-eyed groups and normals but also between SS and KCS. Both total tear film protein and lipocalin concentrations were significantly lower in SS compared with both NDE and KCS, whereas lysozyme concentration was similar in all three groups. To our knowledge, this is the first report detailing total tear protein, lipocalin and lysozyme concentrations in multiple AD dry-eyed groups, and our data suggest that a combination of tear flow and

protein data can provide valuable information to assist in the differential diagnosis of various forms of dry eye. This study was conducted as a pilot on a small number of patients. Not all SS subjects (5/25) were able to provide sufficient material for full biochemical analysis. Specifically, total tear protein collected in the eye wash was insufficient, either because of very low protein concentration of the tears or low recovery volume. It is our experience that on very dry eyes, a large fraction of the applied saline is absorbed by the ocular surface. Thus, given the potential relevance of our findings, further investigation of these biomarkers is suggested.

By definition, mean Schirmer I scores from both SS and KCS groups were significantly reduced compared with NDE; however, no difference was found between the two dry-eyed groups. Although our findings agree with previous work,<sup>3,5</sup> some studies have discriminated KCS from SS based on Schirmer scores.<sup>25</sup> Thus, while tear flow may prove useful in a broad separation of aqueous deficient dry eye from non-dry-eyed subjects, standard practice suggests that the combination of several tests, including biomarker quantitation, be performed to increase the sensitivity and selectivity required for adequate differential diagnosis.<sup>3,18,20</sup>

Age may have been a factor in our results. The mean age of the two dry-eyed populations was greater compared with the control group, with significance reached in the SS group. We cannot totally rule out that this age difference may have influenced our findings, as the literature does suggest that tear volume, production stability and/or quality is reduced in the older population.<sup>26-29</sup>

Our data agree with previous studies that have demonstrated altered SS tear biochemistry relative to control populations.<sup>3-5,30</sup> Tear lipocalin<sup>31-35</sup> is a member of the

lipid binding protein super-family and possesses antimicrobial activity in addition to its ability to scavenge lipid products of inflammation.<sup>36-38</sup> Surface pressure measurements of human tears with individual tear components indicate that proteins are major contributors to surface pressure and can significantly influence tear stability.<sup>39</sup> Thus we can speculate that a reduction in lipocalin and/or total tear protein could potentially compromise both the function and integrity of the tear film in subjects with SS, further contributing to the damage associated with reduced amounts of aqueous in the tear film. In addition, the different protein profiles in SS and KCS groups support the theory that the pathology underlying AD dry eye involves more than just a failure to secrete aqueous. The fact that the KCS group had a diminished ability to secrete aqueous but appeared equal to the NDE group with respect to protein profile suggests multiple mechanisms of secretion and/or alternative protein sources.<sup>46</sup> That no correlation was found between tear flow and protein concentrations supports this hypothesis. However, it is also possible that very minor alterations in other proteins not investigated in this work and not reflected in total protein content are involved in KCS pathophysiology.<sup>2,17,19</sup>

The finding that lysozyme concentration remained invariant across the three populations was not expected. Research on the healthy lacrimal gland suggests tear lipocalin colocalizes with lysozyme<sup>40</sup> and that lipocalin and lysozyme are formed and released in unison.<sup>41</sup> Directly comparing the lipocalin and lysozyme data from individual samples in this study failed to find a correlation between the concentrations of these two proteins. Reduced tear lysozyme has been reported previously in SS<sup>42</sup> and non-SS dry-eyed<sup>43</sup> subjects. Methodological differences<sup>43</sup> may in part account for our findings in that we employed an eye wash technique as opposed to tear collection with a capillary tube. It



is possible that by applying a bolus of sterile saline, we stimulated a reflex secretion of proteins, although previous studies have demonstrated that reflex stimulation does not affect protein profile.<sup>44-45</sup> It is also possible that sites other than main and accessory lacrimal glands are capable of protein production and secretion. Recent proteomic data suggest that a number of proteins, including lysozyme and lipocalin, may be secreted from human meibomian glands.<sup>46</sup> Clearly additional work is needed to characterize the production and function of various tear film proteins in both health and disease.

## **4.6 Conclusions**

This study highlights the unique nature of dry eye disease associated with primary Sjogren's syndrome. It suggests that the lacrimal gland in SS behaves differently from that of a non-SS aqueous deficient dry eye patient. It also suggests that although no one clinical test may be able to provide adequate sensitivity and selectivity for diagnosis, the quantitation of biomarkers may provide support in the diagnosis of SS. These analyses may also enhance our understanding of the pathophysiology of dry eye disease and, in so doing, further the advancement of novel treatments.

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## 5 MUC16 Expression in Sjogren's Syndrome

This chapter is the basis of a paper published in 2008: Caffery B, Joyce E, Heynen M, et al. MUC16 expression in Sjogren's syndrome, KCS, and control subjects. *Mol Vis* 2008;14:2547-55.

### 5.1 Overview

**Purpose:** To investigate the expression of MUC16 protein in tears and conjunctival cell membranes and MUC16 mRNA in conjunctival cells of Sjogren's syndrome (SS), keratoconjunctivitis sicca (KCS) and non-dry-eyed (NDE) subjects. The relationship of tear flow and soluble MUC16 concentration was also measured.

**Methods:** Seventy-six (76) subjects were recruited for this study: 25 SS (confirmed via American-European Consensus Criteria 2002), 25 KCS (confirmed by symptoms and Schirmer scores  $\leq$  10mm) and 26 NDE. Tear flow was measured by the Schirmer test without anesthesia for 5 minutes. Tears were collected using an eye-wash technique. Protein and mRNA were isolated from conjunctival epithelial cells collected via impression cytology. Soluble and membrane bound MUC16 were quantified via Western blotting and MUC16 mRNA was quantified by real time qPCR.

**Results:** The SS group demonstrated significantly higher concentrations of soluble MUC16 (7.28 [SS] $\pm$ 3.97 vs 3.35[KCS] $\pm$ 4.54 [p=0.004] and vs 1.61 [NDE] $\pm$ 1.22 [p<0.001]) and MUC16 mRNA (4.66 [SS] $\pm$ 5.06 vs 1.84[KCS] $\pm$ 2.26 [p=0.01] and 1.52 [NDE] $\pm$ 1.04 [p=0.003]) compared with both KCS and NDE groups, respectively. No

differences in soluble or mRNA MUC16 were found between the KCS and NDE groups. Membrane bound MUC16 was similar in all three groups. No significant correlation was found between mean Schirmer values and any measure of MUC16 expression.

**Conclusions:** These results demonstrate that SS subjects display a significant increase in both soluble MUC16 and MUC16 mRNA concentrations compared with other forms of aqueous deficient dry eye and non-dry-eyed individuals. There was no correlation between tear flow and soluble MUC16 concentration.

## 5.2 Introduction

Mucins constitute an important part of the precorneal tear film and ocular surface. Our current understanding is that the tear film is best described as a mucin/aqueous gel decreasing in density toward the lipid layer.<sup>(1, 2)</sup> Mucins are believed to play a key role in the retention of water and other tear fluid components on the ocular surface, hence maintaining both lubricity of the ocular surface and a healthy epithelial barrier.<sup>(3, 4)</sup> Early concepts of ocular mucins described goblet cells as the sole origin of secretion.<sup>(5)</sup> However, numerous studies have now shown multiple species of mucins are also derived from the ocular surface epithelium.<sup>(6-10)</sup>

To date, 20 human mucin genes have been completely or partially sequenced. They have been named and numbered chronologically with their discovery: MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6-9, MUC11-13 and MUC15-20.<sup>(11-19)</sup>

Based on the presence of common structures within their amino acid sequences, they have been grouped together into three broad groups:<sup>(6, 11, 14-17, 20-22)</sup>

1. Gel-forming mucins from goblet cells of various epithelia: MUC2, MUC5AC, MUC5B, MUC6, MUC19
2. Soluble mucins: MUC7, MUC9
3. Membrane-associated mucins: MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20.

Both secreted (MUC2, MUC5AC, MUC5B, MUC7) and membrane bound (MUC1, MUC4, MUC16) mucin forms have been reported to be expressed by ocular



surface epithelia.<sup>(6, 23, 24)</sup> Of the mucins identified on the ocular surface, two soluble (MUC2 and MUC5AC) and three membrane-bound (MUC1 and MUC4, MUC16) forms are considered “critical” for the maintenance of a “normal” tear film.<sup>(23)</sup> MUC2 and MUC5B are present in very low quantities.<sup>(23-25)</sup>

Recent research has demonstrated that alternative soluble forms<sup>(25-29)</sup> of MUC1<sup>(26)</sup> and MUC16 exist, as has been illustrated by Spurr-Michaud et al.,<sup>(25)</sup> who described soluble MUC16 in the tears. The alternative forms of MUC1 and MUC16 lack the cytoplasmic tail portion of the protein and thus are secreted or shed into the tear film, as opposed to being anchored on epithelial cell membranes. Whether these soluble species are present in all tear samples or are linked to ocular surface pathology is not yet clear. Also unclear are the functions attributed to these alternative, soluble forms of MUC1 and MUC16.

The functions of MUC16 are slowly becoming understood. Blalock et al., using immortalized corneal-limbal epithelial cells (HCLE), suggested that MUC16 forms a protective barrier on these cells, without which there is rose bengal dye penetrance and adherence of *S. aureus*.<sup>(24)</sup> They locate MUC 16 on the tips of the HCLE cell surface and suggested that the cytoplasmic tail binds to the actin cytoskeleton. No such work has been done with human conjunctival cells.

Although alteration in mucin expression and/or mucin glycosylation have been implicated in the pathophysiology of dry eye, only a limited number of studies addressing these issues have been conducted. The recent DEWS report eliminated the separate classification of primary mucin deficient dry eye,<sup>(30)</sup> with their classification supporting both aqueous-deficient (AD) and evaporative (E) dry eye groups. Within the AD dry eye

group, two major subclasses exist, namely Sjogren's syndrome (SS) dry eye and non-SS dry eye.

The role of mucins in dry eye disease has mainly focused on MUC5AC, and it is generally assumed that the expression of MUC5AC is reduced in dry-eyed subjects.<sup>(31)</sup> Recently, attention has also been placed on membrane spanning mucins and their potential role in dry eye.<sup>(24, 32)</sup> Danjo et al. have reported that the conjunctival epithelial cell distribution of H185 (MUC16) is altered in non-SS dry eye subjects.<sup>(32)</sup>

It is the membrane spanning mucin MUC16 that is the subject of this paper. Specifically, we sought to characterize the expression of MUC16 in Sjogren's syndrome dry eye as compared with aqueous deficient (KCS) dry eye and non-dry-eyed (NDE) controls, to gain further insight into the role that MUC16 may play in dry eye disease. In addition, we examined the relationship between soluble MUC16 expression and tear flow as measured by Schirmer testing.

## **5.3 Materials and Methods**

### **5.3.1 Study design**

Prior to the start of this study, ethics approval was attained from the Office of Research Ethics at the University of Waterloo and the University Health Network, and all procedures adhered to the Declaration of Helsinki. A total of 76 subjects – 26 non-dry-eyed controls (NDE), 25 Sjogren's subjects (SS) and 25 non-Sjogren's keratoconjunctivitis subjects (KCS) – were enrolled in this study. All participants underwent a clinical evaluation visit to determine entry eligibility, prior to a second visit in which ocular samples were collected.

All SS participants had been diagnosed with primary SS at the Sjogren's Syndrome Clinic of the University Health Network, using the American-European consensus criteria of 2002.<sup>(33)</sup> Thus, each of these subjects had 3 or more of the following criteria: symptoms and signs of dry eye and dry mouth as well as either a positive minor salivary gland biopsy or the presence of antibodies to Ro and/or La. Recruitment of these patients was achieved through telephone calls from the database of that clinic. No further preliminary screening was performed on this group as all had confirmed Sjogren's syndrome.

The KCS and NDE subjects were recruited through the SS clinic and a private practice. Participants first answered the question, "If you have dry eyes, have they been dry for at least 3 months?" Participants who answered yes were asked to rate their dryness on a visual analogue scale used routinely in the SS clinic. The horizontal line of the scale was marked from 0 to 10. At the 0 point the phrase "not dry at all" was written and at the 10 point "as dry as the desert" was written. If they rated their dryness as greater than or equal to 6 out of 10 on the visual analogue scale and the Schirmer 1 test score was less than or equal to 10 mm in 5 minutes in at least one eye, they were classified as KCS. Non-dry eye subjects (NDE) were enrolled if they stated that they did not have dry eyes, ranked their dryness as 0 on the visual analogue scale and had Schirmer I scores of greater than 10 mm in both eyes. All subjects were free from allergy or other ocular surface diseases, and all were on maximum therapy for blepharitis, if that condition had been previously diagnosed. Thus, where appropriate, they were using lid scrubs and hot soaks but were not using topical antibiotics or topical anti-inflammatories. Pre-screening for KCS and NDE subjects was performed within two months of the actual clinic visit for

collection of tears and impression cytology specimens. Participants were required to confirm that their dry eye status had not changed at the collection visit to be included in the study.

### **5.3.2 Reagents and materials**

Agarose was purchased from Cambrex Bio Science (Rockland, ME, USA). Gel buffer, tank buffer, vacuum blotter, nitrocellulose membrane and blotting paper were purchased from BioRad (Mississauga, ON, Canada). Glycerol and 20x SSC transfer buffer were purchased from VWR (Mississauga, ON, Canada). Molecular weight standards (Himark™) were purchased from Invitrogen (Carlsbad, California, USA). ECL-Plus™ kits were purchased from GE Healthcare (Baie d'Urfe, QC, Canada). DC Protein Assay Kits® were purchased from BioRad Laboratories (Mississauga, ON, Canada). Monoclonal mouse anti-human MUC16 antibody (OC125) was purchased from DAKO (Glostrup, Denmark) and goat anti-mouse IgG-HRP from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). Millipore™ Membrane Filters were purchased from Millipore™ (Billerica, MA, USA)

### **5.3.3 The Schirmer I test**

The Schirmer I test was performed using pre-packaged, sterile paper strips (Schirmer Tear Test Strips®, Alcon, Fort Worth, TX), without anesthesia. The rounded bulb end of the strip was folded at the notch and then inserted into the lower fornix, one third of the distance from the lateral canthus of the lower lid. Once both strips were in place, the subject was asked to close his or her eyes. After 5 minutes the strips were removed and the wet portion measured in millimetres.

### **5.3.4 Eye wash tear collection**

Tears were collected using an eye wash method as described elsewhere.<sup>(31)</sup> Briefly, 60  $\mu$ l of sterile, physiological saline (0.9% NaCl) (Minims, Chauvin Pharmaceuticals Ltd, Romford, Essex, UK) was applied to the superior bulbar region of the unanaesthetized ocular surface (right eye) using a sterile micropipette. Participants were asked to rotate their eyes without blinking (lids were still held open), to mix the tear fluid. Tear washes were collected from the inferior fornix of each eye using the sterile micropipette. The same procedure was repeated with the left eye. Both eye washes were pooled together, vortexed briefly, and then placed on dry ice until transfer to  $-80^{\circ}\text{C}$  for storage.

### **5.3.5 Conjunctival impression cytology (CIC)**

Conjunctival epithelial cells were collected via impression cytology from each eye using sterile Millipore, MF membranes (pore size 0.45  $\mu\text{M}$ ). Two drops of a topical anaesthetic (Alcaine®, Alcon), dosed 60 seconds apart, were applied to the right eye. Fifteen (15) seconds after the second drop of anaesthetic, the subject was instructed to hold gaze down to expose the superior conjunctiva. The investigator held the upper lid up to fully expose the superior conjunctiva. One piece of filter paper was placed on the superior region of the conjunctiva for five to seven seconds then removed with blunt forceps and placed in a sterile pre-labelled 2 ml capped polypropylene centrifuge tube containing 1 mL of RLT® RNA Isolation Buffer (Qiagen, Maryland, USA) with 0.01%  $\beta$ -mercaptoethanol. The same procedure of impression cytology then took place on the temporal conjunctiva and the filter paper was placed in the same tube as the superior sample. Anaesthesia and impression cytology of the left eye then took place as described

for the right eye, with the exception that the two filter papers were placed in an empty, sterile 2 ml capped polypropylene centrifuge tube, such that protein extraction could take place. All samples were immediately placed on dry ice and then transferred to -80°C for storage until processing.

### **5.3.6 Protein isolation from CIC samples**

Impression cytology filter papers that were collected from the left eyes of subjects were used to isolate total protein. Filter papers were placed cell side up on small glass plates and 5 µL of extraction buffer (2% SDS; 1X Complete™ protease inhibitor cocktail [Roche, Mannheim, Germany]) was placed on each. Using a steel scalpel blade, each membrane was cut into small pieces, which were placed in 600 µL capped polypropylene centrifuge tubes and covered with an additional 50 µL of extraction buffer. Tubes were vortexed and then heated at 95°C in a heating block for 10 minutes. Tubes were centrifuged at 12 000 g for 6 minutes to pellet the filter pieces, and the protein extract was collected and transferred to a fresh capped polypropylene centrifuge tube. Twenty microlitres (20 µL) of extraction buffer was added to the pelleted filter paper. Following vortex and centrifugation, wash was collected and added to the first protein aliquot.

### **5.3.7 Determination of total protein concentration in tear and CIC samples**

All total protein determinations were conducted using the DC Protein Assay Kit® (BioRad), following manufacturer's instructions. For samples that contained SDS, 20 µL of Reagent S was added to each ml of Reagent A. Five microlitres (5 µL) each of eye wash or impression cytology extract was added to 5 µL of Milli-Q water, and the final 10

$\mu\text{L}$  was divided equally between two microplate wells to allow assay in duplicate. Absorbances were read at 750 nm on a Multiskan Microplate Spectrophotometer.

### **5.3.8 Electrophoresis and immunoblotting**

Samples were thawed at room temperature and diluted 4:1 with 5X sample buffer concentration :247 mM Tris-HCl, pH 8.6, 2% SDS (w/v), 50 mM DTT, 1X Complete™ Protease Inhibitor (Roche, Mannheim, Germany), 10% glycerol, 0.002% (w/v) bromophenol blue. Samples were further diluted with 1X sample buffer to achieve a final protein concentration of 1  $\mu\text{g}/\mu\text{L}$ . Samples were heated at 100°C for 3 minutes, cooled to room temperature and then placed on ice. MUC16 standard antigen (CA125; 1 – 60 Units/well) was run on each gel to normalize data and facilitate semi-quantitation of samples through linear regression analysis. Ten (10)  $\mu\text{g}/\text{lane}$  of eye wash protein and 5.0  $\mu\text{g}/\text{lane}$  of CIC total protein were loaded per lane. Following separation, protein was transferred to nitrocellulose membranes via vacuum transfer with 4x SSC buffer for 2 hours. Membranes were fixed by heating at 70°C for 30 minutes. Membranes were air dried for 12 hours and then blocked in PBS + 0.05% Tween 20 (= PBS-T) + 0.1% BSA + 10% NAP (NAP-Blocker, G Biosciences. Maryland Heights, USA), for 1 hour at room temperature on an orbital shaker. Following three washes with PBS-T, blots were incubated overnight in mouse monoclonal antibody clone OC125 (1:250) in PBS-T and 0.1% BSA + 10% NAP at 4°C. After rinsing in PBS-T, blots were incubated with the secondary antibody (1:5000) in PBS-T + 0.1% BSA + 10% NAP for 1 hour at room temperature. Blots were developed with ECL® (BioRad) and chemiluminescent signals were captured by Storm840® Imaging (Molecular Dynamics). The amount of MUC16 in each sample and standard were quantified by image analysis software (ImageQuant 5.1®),

Molecular Dynamics). Known amounts of CA125 standard were used to generate standard curves, and using the line-of-best-fit from the standard curve, the relative amount of mucin in the samples was interpolated from the graph. It should be noted that samples often produced multiple chemiluminescent signals of varying molecular weights. For quantitation, only signals above 300 kDa for MUC16 were used.

### **5.3.9 RNA Isolation from CIC Samples and Reverse Transcription**

Tubes containing 1 mL of RLT® buffer (Qiagen) and two impression cytology samples collected as described above were allowed to thaw at room temperature and then vortexed for 30 seconds. Membranes were removed using a 21 gauge needle and samples were vortexed again and then passed through a 21 gauge needle 10 times. Extraction of total RNA proceeded according to manufacturer's directions (RNeasy® Minikit, Qiagen). The DNase step, as recommended, was performed. The final isolation step was conducted with 40 µL of RNase free water. Following a 1 min centrifuge step (8000g), flow-through (total RNA) was collected and stored at -80°C.

RNA quantity and quality were assessed by measuring the optical density using a Beckman DU530 Life Science UV/Visible Spectrophotometer at 260 nm and 280 nm. DNA was synthesized from 8 µL of RNA sample using random hexamer primers with Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

### **5.3.10 Real Time qPCR**

Relative expression of genes of interest was performed in multiplex PCR reactions containing target (MUC16) and endogenous control (GAPDH) oligonucleotide primers in the presence of gene-specific dye-labelled Taqman probes (Table 5.1). Two



μL of cDNA was used for amplification in a 50 μL PCR reaction containing target (300 nM) and endogenous control (100 nM) oligonucleotide primers, control and target Taqman probes (100 nM), and Taqman<sup>®</sup> Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Duplicate samples were used for analysis in a 7500 Real Time PCR System (Applied Biosystems). Conditions used for amplification were as follows: 50°C for 2 minutes, followed by an initial 10 minute denaturing step at 95°C. This was followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds. Normalized reporter dye fluorescence ( $R_n$ ) data were collected during the extension step at each cycle.

**Table 5.1: Sequence data for gene amplification in real time RT-PCR**

Gene	Forward Primer	Reverse Primer	Taqman Probe
MUC16	ACCCAGCTGCAGAACTTCA	GGTAGTAGCCTGGGCACTGT	6FAM- GCGGAAGAAGGAAGGAGAAT
GAPDH	GAAGGTGAAGGTCGGAGTCA	GACAAGCTTCCCGTTCTGAG	VIC-CAATGACCCCTTCATTGACC

Collected data were analyzed and fold-expression changes were calculated using the comparative method ( $2^{-\Delta\Delta CT}$ ) of relative quantification by SDS software (v1.3.1; Applied Biosystems). A sample containing 0.25 pg of plasmid DNA with cloned target and endogenous fragments was used as a calibrator sample for each gene.

### 5.3.11 Data analysis

Statistical analysis was performed using Statistica Ver7.1 (StatSoft Inc., Tulsa, OK, USA) and Microsoft Excel<sup>™</sup> XLfit© software. Graphs were plotted using Statistica Ver7.1. All data are reported as mean ± standard deviation. Statistical differences

between groups for biomarker data were identified by using one-way ANOVA, and when necessary, Dunnett's comparison of means and by Tukey's test. Significance was identified at  $p < 0.05$  ( $\alpha = 0.05$ ).

## 5.4 Results

### 5.4.1 Demographics and Tear Flow Measurements

A total of 76 subjects were enrolled into this study, and the subject demographics are displayed in Table 5.2.

**Table 5.2: Summary of demographic information for study groups**

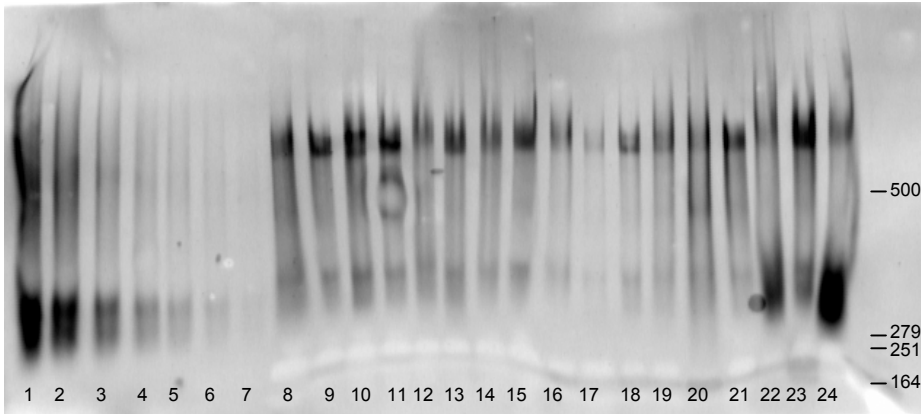
Group	Mean Age (years)	Number of Female Subjects	Number of Male Subjects	Total Subjects
Control Group of Non Dry-Eyed	52.4 ± 11.4	24	2	26
KCS	59.3 ± 9.1	21	4	25
Sjogren's Syndrome	60 ± 11.8*	21	4	25

- denotes significantly greater compared to control group ( $p = 0.024$ )

The mean age of the SS group was found to be statistically higher than the NDE group ( $p=0.024$ ) but not different from the KCS group ( $p>0.05$ ). Mean Schirmer I scores from both eyes collected without anaesthesia for five minutes revealed a significantly reduced ( $p<0.0001$ ) tear flow in both SS ( $5.12 \pm 5.96$  mm) and KCS subjects ( $7.84 \pm 7.35$  mm), relative to NDE ( $23.83 \pm 7.85$  mm). There was no difference in mean Schirmer I scores between the KCS and SS groups ( $p = 0.19$ ).

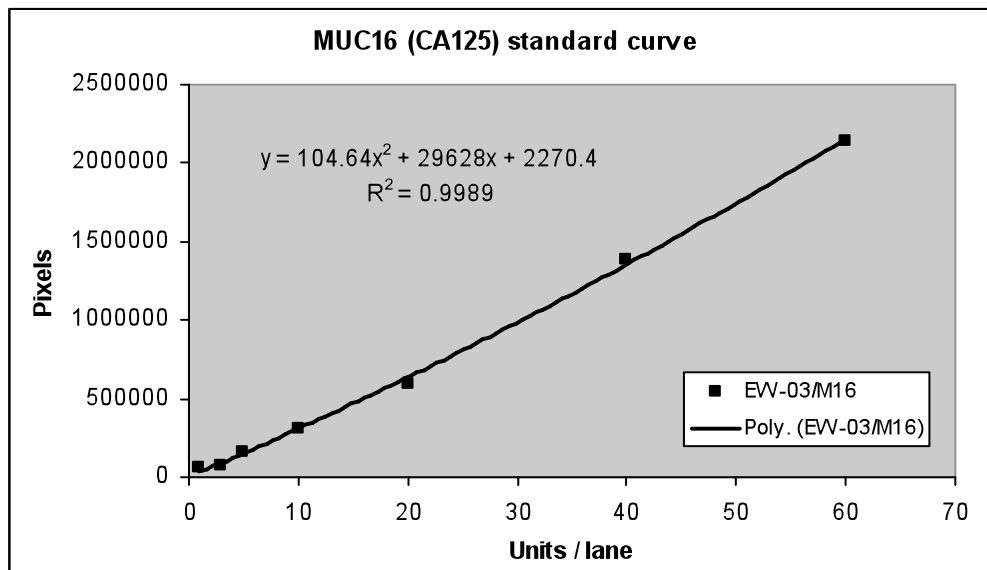
Five of the 25 SS subjects did not supply sufficient tear samples for analysis of soluble MUC16, limiting this analysis to 20 of the 25 subjects. Representative data is displayed in Figure 5.1. All samples that were analyzed displayed quantifiable amounts of soluble MUC16, although significant inter-sample variation was observed both in terms of migration pattern of the MUC16 signal on Western blots (Figure 5.1) and total amount of MUC16 (Figure 5.3). Mean data showed that the SS group demonstrated significantly higher concentrations of soluble MUC16 compared with both KCS ( $7.28 \pm 3.97$  vs  $3.35 \pm 4.54$ ;  $p=0.004$ ) and NDE ( $7.28 \pm 3.97$  vs  $1.61 \pm 1.22$ ;  $p<0.0001$ ) groups (Figure 5.4). The SS group also demonstrated a significantly higher concentration of MUC16 mRNA compared with both KCS ( $4.66 \pm 5.06$  vs  $1.84 \pm 2.26$ ;  $p=0.01$ ) and NDE ( $4.66 \pm 5.06$  vs  $1.52 \pm 1.04$ ;  $p=0.003$ ) groups (Figure 5.5). No differences in the concentrations of soluble MUC16 and MUC16 mRNA were found between the KCS and NDE groups ( $p>0.05$ ). A weak ( $r^2=0.13$ ) but significant correlation was found between the expression of soluble MUC16 protein and mRNA. Lastly, no difference between membrane bound MUC16 was found between any groups ( $p>0.05$ ).

**Figure 5.1: Representative Western blotting data**



An example of a Soluble MUC16 Western blot from tear samples derived from 17 subjects. Lanes 1 to 7 are MUC16 standard antigen (CA125) Units (based on radio-immunoassay calibration from the vendor); (Lane 1 = 60, Lane 2 = 40, Lane 3 = 20, Lane 4 = 10, Lane 5 = 5, Lane 6 = 3, Lane 7 = 1 U); Lanes 8 to 24 are tear samples.

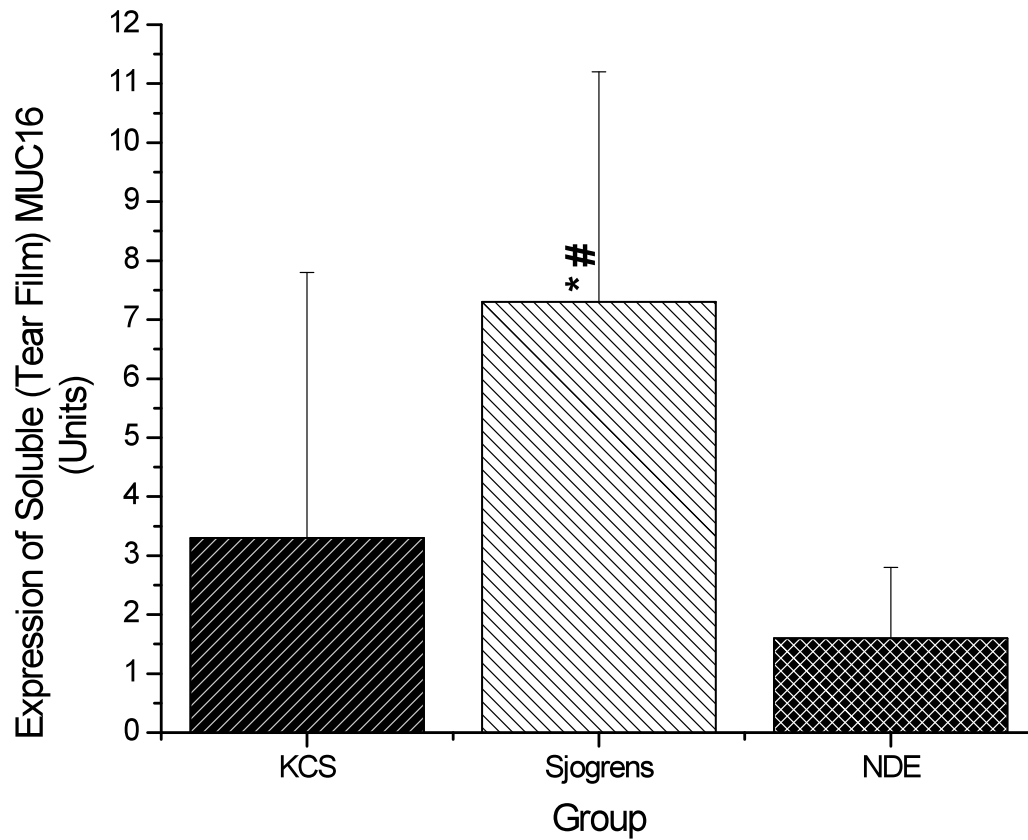
**Figure 5.2: Western blot and regression analysis for soluble MUC16 quantification**



A regression curve was created by graphing applied concentration of CA125 standard against the optical density of the resulting band immunoreactivity. Total MUC16 concentration was quantified by extrapolation from this curve using all signal above 300 kDa.

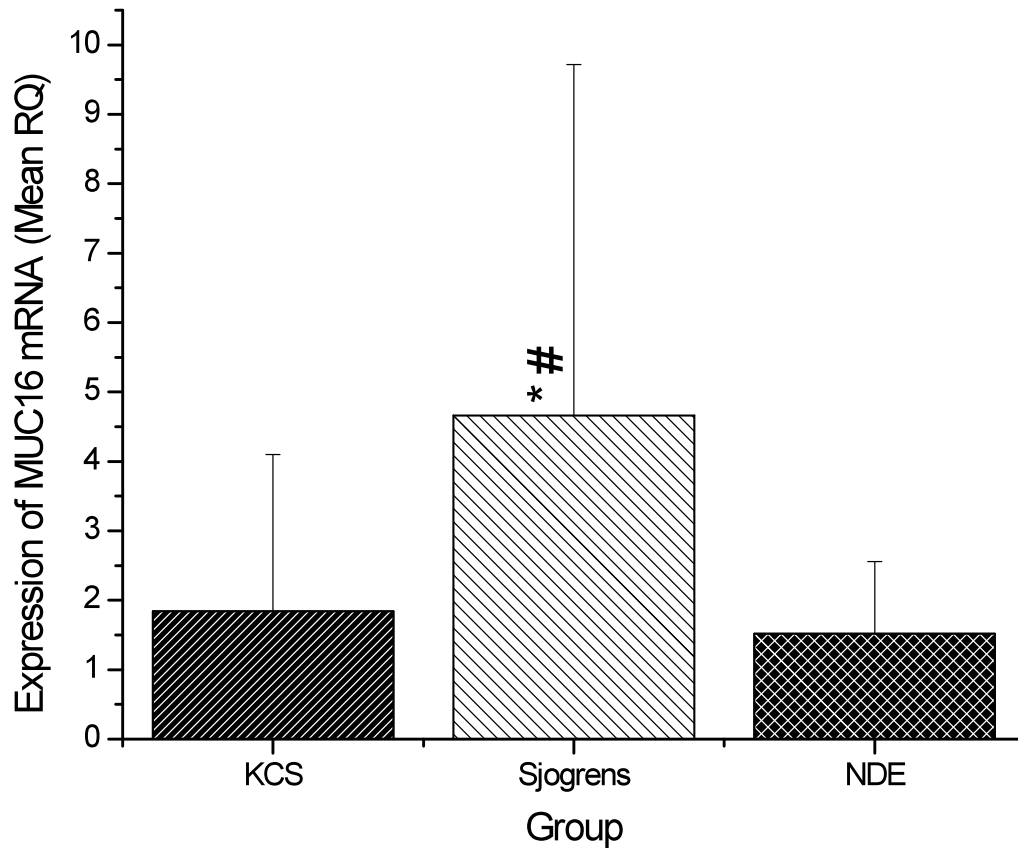


**Figure 5.4: Summary of Soluble MUC16 as Quantified by Western Blotting: Mean data**



Soluble MUC16 Expression as Quantified by Western Blotting: Data expressed as mean data. Protein samples collected via eye wash and MUC16 data expressed in Units as calculated from extrapolation from a standard curve titration of CA125. (\*) = significantly different compared to NDE Group (#) = significantly different compared to KCS Group.

**Figure 5.5: Summary of MUC16 mRNA Expression as Quantified by qPCR**



RNA isolated from conjunctival epithelial cells collected via impression cytology. (\*) = significantly different compared to NDE Group; (#) = significantly different compared to KCS Group.

A single significant correlation ( $p=0.03$ ) was found between mean Schirmer values compared with any measure of MUC16 expression; that being soluble MUC16 concentration in the combined KCS and NDE groups (Table 5.3).

**Table 5.3: Slope and correlation data summary for comparison of MUC16 expression data with mean Schirmer scores**

<b>Group</b>	<b>Soluble MUC16 vs Schirmer Score</b>	<b>Membrane Bound MUC16 vs Schirmer Score</b>	<b>MUC16 mRNA vs Schirmer Score</b>
Sjogren's Data (n=20)	$y = 0.02x + 7.15$ $r^2 = 0.001; p = 0.87$	$y = 0.22x + 7.73$ $r^2 = 0.02; p = 0.46$	$y = 0.05x + 4.47$ $r^2 = 0.004 p = 0.77$
KCS (n=25) and NDE (n=26)Data	<b><math>y = -0.1x + 4.04</math></b> <b><math>r^2 = 0.09; p = 0.03</math></b>	$y = -0.04x + 7.9$ $r^2 = 0.01; p = 0.47$	$y = 0.004x + 1.6$ $r^2 = 0.0005; p = 0.88$

## 5.5 Discussion

The results of this study demonstrate that subjects with confirmed Sjogren's syndrome display a significant increase in MUC16 expression, as the concentrations of both MUC16 soluble protein and mRNA were found to be higher than those of aqueous deficient dry eye subjects (KCS) and subjects with no dry eye disease (NDE). There were no differences found in membrane bound MUC16 protein concentration between any of the groups and no difference in any form of MUC16 found between the KCS and NDE groups. Lastly, no correlation between tear flow and MUC16 expression was found in any group.

The numbers of subjects in this pilot study were small. Not all SS subjects (20/25) were able to provide sufficient material for full biochemical analysis. Specifically, total tear protein collected in the eye wash was insufficient, either because of very low protein concentration of the tears or low recovery volume. It is our experience that on very dry eyes a large fraction of the applied saline is absorbed by the ocular surface. Thus, given



the potential relevance of our findings, further investigation of MUC16 in larger groups is warranted.

We note that age may have been a factor in our results. The mean age of the two dry-eyed populations was greater compared with the control group, with significance reached in the SS group. We cannot totally rule out that this age difference may have influenced our findings, as the literature does suggest that tear volume, production stability and/or quality are reduced in the older population.<sup>(34, 35)</sup> It is not known how mucin changes with age on the ocular surface. However, in our study, there was no significant age difference between the KCS and SS groups that did show differences.

This group of primary Sjogren's syndrome patients was well-defined, as the diagnosis was confirmed by the American-European consensus criteria of 2002.<sup>(33)</sup> Our KCS group had Schirmer test-confirmed reduced aqueous secretions, and thus we were able to compare the two well-defined aqueous deficient groups as stated in the DEWS 2007 definition of dry eye.<sup>(30)</sup>

Others have explored transmembrane mucin expression in SS and dry eye disease. Data reported by Spurr-Michaud et al. describing the presence of MUC16 in the tear film is confirmed by our results.<sup>(25)</sup> That MUC16 is one of the transmembrane mucins of the surface conjunctival epithelium was determined by Argueso et al.,<sup>(9)</sup> and its presence within the conjunctival epithelium in both protein and mRNA is confirmed with this study. There is, however, very little additional work on MUC16 in SS. Data on other membrane bound mucins has suggested that the expression of mucosal epithelial membrane mucin (as detected by an uncharacterized antibody referred to as AMEM2) is reduced in SS and non-SS dry-eyed subjects compared with controls.<sup>(36)</sup> At the genetic

level, a specific splice-variant of the MUC1 gene may be reduced in dry-eyed subjects,<sup>(37)</sup> although other authors have failed to find differences in MUC1 or MUC4 gene expression between controls and SS subjects.<sup>(31)</sup>

Our finding of excess MUC16 on the SS ocular surface was particularly interesting to the clinical author (BC), who has long observed that excess ocular mucus is a common clinical finding in SS patients. The sole reference to excess mucus in SS that we have noted is that of “mucous aggregates” adherent to the conjunctival and corneal epithelia in a paper by Pflugfelder et al. in 1990.<sup>(38)</sup> To our knowledge, this is the first report of an increase in MUC16 in the tear film of SS subjects. The authors also understand that the literature does not characterize SS as a disease of excess mucus production. In fact, most other papers have suggested that dry eye disease is a disease of reduced mucus production and secretion.<sup>(36, 39, 40)</sup> Certainly, the secreted mucin MUC5AC has been shown to be reduced in SS tears and conjunctival cells.<sup>(31)</sup>

Alterations in the distribution of conjunctival epithelial MUC16 have been noted in dry eye disease. Danjo et al., using immunolocalization, noted that superficial temporal conjunctival epithelial cells did not bind the H185 antibody (MUC16) in non-SS dry eye as well as in normal subjects.<sup>(32)</sup> Our Western blot analysis failed to support these findings. The H185 epitope has been established as MUC16; however, we cannot rule out that different detection probes may have influenced these results and that the identification of MUC16 may be influenced by the level of glycosylation. That differential glycosylation of MUC16 may be a relevant area of study is demonstrated by the significant heterogeneity of signal migration as evidenced in Western blots. Whether

differential glycosylation affects antibody recognition and/or the function properties of MUC16 protein is not known currently.

The selection of dry-eyed subjects is an important variable in comparing these studies. In part I of Danjo's study,<sup>(32)</sup> when differences between dry eyes and normals were studied, subjects were chosen by dry eye symptoms and the presence of rose bengal or fluorescein staining of the ocular surface, in the absence of autoimmune disease. As Schirmer testing was not part of this group's inclusion criteria, true aqueous deficiency was not established. KCS subjects in the present study were enrolled based on the presence of at least moderate dry eye symptoms and a Schirmer score of less than or equal to 10 mm. Another notable difference is that staining was not an inclusion criterion in our study. Such differences in inclusion criteria make comparisons between published studies somewhat difficult.

Which epithelial cells were used for characterization is also a relevant factor. In this study impression cytology was performed on both temporal and superior bulbar conjunctival areas, and the samples were pooled. Danjo et al., in part I of their study, used cells from the temporal conjunctiva in the dry eye group and a combination of inferior and temporal conjunctiva from the normal controls. Clinically, staining patterns of the conjunctiva are quite different and as such, it is likely that differential expression of various mucin species may be a function of which cells were analyzed. Comparing mucin expression in exposed and non-exposed conjunctival cells would be an important contribution to the literature.

Alterations in membrane MUC16 have been studied in relationship to conjunctival staining. Dry eye patients, particularly those with Sjogren's syndrome,

present clinically with rose bengal staining of the conjunctiva and cornea.<sup>(33)</sup> Initially rose bengal was thought to stain cells that were desquamated or dead.<sup>(41-44)</sup> More recently, the dysfunction of mucins has been implicated. Danjo et al.<sup>(32)</sup> studied aqueous deficient but not SS dry-eyed subjects, who had Schirmer scores of less than or equal to 5 mm in 5 minutes and staining by rose bengal or fluorescein, in part II of their study. They found a significant correlation between staining scores of the temporal conjunctiva and an altered H185 (MUC16) binding pattern. Two studies used a human corneal-limbal epithelial cell line (HCLE) to demonstrate that MUC16 surface protein protects against rose bengal invasion.<sup>(24, 28)</sup> Others have reported a positive correlation between decreased transmembrane mucin (not identified) and higher rose bengal staining in aqueous deficient dry eye.<sup>(36)</sup> Although we did not stain the ocular surface of the subjects at the time of their visit for tear and cell collection in this study, our results suggest that staining scores, at least in SS patients, would not correlate with a reduction in membrane MUC16, as we found no differences in membrane bound MUC16 expression in any of our subject groups.

The observation of excess ocular mucus in SS patients and the results of this study that show excess MUC16 in the tear film of SS subjects allowed us to speculate on the possible mechanisms of such mucin production. It appears that there is an active upregulation of mucin production in our SS subjects, as determined by increased mRNA, followed by excess shedding of this mucin into the tear film. Mucin production in humans is an ancient defence mechanism,<sup>(45)</sup> and non-ocular mucous membranes, such as those of the airways, demonstrate excess mucus production under adverse conditions in dogs, rats and humans.<sup>(46-48)</sup> The ocular surface performs compensatory mucin related

activities in other autoimmune states such as ocular cicatricial pemphigoid (OCP). As the OCP ocular surface moves towards keratinization, there is increased expression of the family of glycosyltransferases that act at the initial stages of mucin glycosylation.<sup>(49)</sup> That these findings were found in the early stages of the keratinization process suggests that ocular surface cells can participate in compensatory attempts to synthesize more mucin to maintain a wet surface phenotype. Since our results demonstrated no correlation between tear flow and MUC16 concentration, we believe that it is the unique nature of autoimmune related dry eye that influences the stimulus for MUC16 expression. Perhaps this is a result of a signalling mechanism peculiar to SS that functions to maintain a more “healthy” ocular surface in the absence of aqueous tears.

Another factor that could increase the concentration of MUC16 on the SS ocular surface is the change in flushing and clearing mechanisms that occur with extreme dryness. Berry et al. suggest that in dry eye, tear mucins may form an irreversible complex with other surface components, which prevents normal removal.<sup>(50)</sup>

## **5.6 Conclusions**

In conclusion, Sjogren’s subjects express significantly elevated concentrations of both soluble MUC16 and MUC16 mRNA compared with both KCS and NDE groups. No differences were found in MUC16 expression between the KCS and NDE subjects. All three groups had similar concentrations of membrane bound MUC16. No correlation was found between tear flow and MUC16 expression. It is probable that conjunctival cells in Sjogren’s syndrome increase their production of MUC16 as a compensatory mechanism to maintain their healthy phenotype.

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## 6 Summary

### Specific findings of this thesis:

1. Rose bengal staining of the conjunctiva is the most distinctive ocular finding in SS, and it best differentiates SS from aqueous deficient dry eye.
2. Staining of the conjunctiva and cornea are distinct variables that complement but do not overlap in SS.
3. Total protein and lactoferrin are found in lower concentrations in the tear films of SS subjects compared with aqueous deficient dry eye and normal subjects. Tear lysozyme is found in the same concentration in all three groups.
4. MUC16 is found in higher concentrations in the tear film of SS subjects, and its mRNA is found in higher concentrations in the conjunctival cells of SS subjects compared with aqueous deficient dry eye and normal subjects.

This research involved a macroscopic view of Sjogren's syndrome through an ophthalmic lens, combined with a microscopic analysis of the tear film and ocular surface mucins. The sum of this work shows that Sjogren's syndrome is a unique form of dry eye disease that is distinguishable in both clinical and laboratory settings from aqueous deficient dry eye. The most intriguing discovery of the large cohort analysis was the importance of conjunctival staining. This observation of rose bengal conjunctival staining, particularly of the temporal area, proved to be the most important ocular variable in distinguishing SS from DE. Factor analysis of the data also showed that conjunctival staining is a separate observation that is distinct from corneal staining. In the past, clinical wisdom suggested that staining of the ocular surface is a continuum. For

example, the criteria for SS require that we combine the corneal and conjunctival staining scores when diagnosing SS.<sup>1</sup> The clear distinction of corneal and conjunctival staining found in the factor analysis suggests that we should look at ocular surface staining in a new light that treats the two tissues separately.

At a molecular level there are clear differences in tear film proteins and surface mucins that distinguish SS and may explain, in part, the increased level of conjunctival staining in SS. The reduced total tear protein and lactoferrin found in the SS subjects may play a role in conjunctival staining. The tear protein analysis also suggests that the lacrimal glands of SS patients function differently from those of dry eye patients.

In an unexpected result, MUC16 was found in higher concentrations in SS than dry eye. In the past dry eye disease has been considered to be a disease of reduced mucin expression.<sup>2-4</sup> The increase in MUC16 that was observed helps to explain the excess mucin strings seen in many SS patients. However, it does little to add to our understanding of increased staining in SS. In fact, the literature suggests that reduced MUC16 expression is one of the mechanisms of rose bengal penetration of conjunctival cells.<sup>5</sup> Although the staining characteristics of the specific SS group studied in the MUC16 research were not obtained prior to the gathering of cells, our large SS cohort showed distinctly more conjunctival staining than did the DE patients. This suggests that there must be mechanisms other than reduced MUC16 expression that explain staining of the conjunctiva in SS. Further research is necessary to determine the differences in the pathophysiology of corneal and conjunctival staining and the changes in mucin secretions of the ocular surface in various forms of dry eye disease.

## **Future research**

The tree analysis in this thesis demonstrates that Sjogren's syndrome is identifiable in the eye care office with simple non-invasive testing. Further tree analysis of dry eye subgroups by age and other variables may help clinicians to more readily identify these SS patients. Working with Sjogren's syndrome patients emphasizes the need for early diagnosis and management. All health care professionals, including eye care providers, need to be aware of the prevalence of the disease and to use the simple techniques of staining, Schirmer testing and quantification of the severity of dry mouth symptoms to identify SS patients. It is noteworthy that Schirmer testing alone, because of its variability, cannot differentiate dry eye from SS and that conjunctival staining is a critical observation. Sjogren's syndrome patients spend a good number of years trying to discover the source of their discomfort,<sup>6, 7</sup> which costs them and the health care system unnecessary time and money. The diagnosis is important to these patients in that there is some relief in knowing that there is a cause for their symptoms. Also, they appreciate being monitored and managed especially because of their increased risk for lymphoma.<sup>8</sup> Since SS begins in salivary and lacrimal glands, and it is these foci of inflammation that perpetuate the disease, early diagnosis may, in the future, lead to early intervention at the exocrine gland level that may modify the disease process.

The natural history of SS and dry eye is not known. Research is required with longitudinal studies to assess how many mild dry eye patients go on to moderate or severe and how many to SS. We need to understand the natural history of SS so that we can relate the likely lifelong experience of this disease to our patients. Also, eye care professionals would benefit from an understanding of the real results of our treatment

plans. Research is needed to understand whether drops and lid care actually make a difference in the ocular outcomes of these patients. Longitudinal studies that monitor staining, Schirmer scores and symptoms would provide valuable information.

Research is also required to understand the differences in corneal and conjunctival staining. Studies that quantify the presence and degree of both forms of staining, in all forms of dry eye, would be useful. Also, the mechanism of staining in both tissues requires further study. There is some difficulty in obtaining human corneal epithelial cells, but the contact lens cytology technique might allow the capture of sufficient cells.<sup>9</sup> In this way we could compare the status of corneal and conjunctival cells in many forms of dry eye disease.

At the clinical level, we do need available tools for conjunctival staining diagnosis. Practitioners do not have liquid rose bengal or lissamine green at their disposal. If strips must be used, then we must find ways to make them work. We need to standardize the methods of dye insertion and observation.

The proteins of the tear film seem an easy target for diagnosis in office. Perhaps simple efficient analysis of tears could be performed routinely in future eye care offices. Determination of which proteins to target is an important consideration. We need longitudinal studies to understand tear film proteins over time, both with age and stage of dry eye disease. This knowledge might lead to topical treatments with specific proteins.

Ocular mucins are also just beginning to be understood. Since impression cytology is a fairly easy procedure to perform in the clinic, more research can be done with this technique. Schirmer strips may serve the purpose quite well. Studies that compare mucin expression in stained versus unstained conjunctival cells and superior and

inferior versus nasal and temporal cells would be of interest. Perhaps the collection of mucus strings in SS and a comparison of their composition with those of allergic subjects would help us to understand the pathophysiology of SS dry eye disease.

In summary, SS is a common disease that requires the attention of eye care practitioners. The routine use of Schirmer and staining tests and the evaluation of dry mouth symptoms will help to identify Sjogren's patients earlier. Particular attention should be paid to those with a pre-existing autoimmune disease such as rheumatoid arthritis, as the prevalence of SS is high in this group. Research should continue in the understanding of the pathophysiology of Sjogren's syndrome dry eye and the unique features that distinguish it from other forms of dry eye disease. These efforts will lead to novel treatments for these patients that will alleviate their symptoms and preserve their ocular surfaces.

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