CLINICAL AND ANALYTICAL STUDIES IN POSTMENOPAUSAL WOMEN SYMPTOMATIC OF DRY EYE

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Introduction

Menopause is defined as a permanent physiological, or natural, cessation of menstrual cycle. It plays an important role in the development of ocular surface dryness symptoms and there is an increased prevalence of dry eye in women, especially those aged over 50. Despite the high prevalence of dry eye in post-menopausal women (PMW), very few studies have been undertaken to understand dry eye disease in a group of PMW who are not on Hormone Replacement Therapy (HRT). Studies in the past on PMW have primarily focused on the relationship between HRT and dry eye. Hence, a series of studies were undertaken to understand the clinical aspects of dry eye and their relationship to a variety of tear film components, in a group of PMW with and without symptoms of dry eye.

The specific aims of each chapter were as follows:

- Chapter 4: To characterize symptoms of dry eye using questionnaires, namely Ocular Surface Disease Index Questionnaire© (OSDI) and the Indiana Dry Eye Questionnaire (DEQ).
- Chapter 5: To characterize clinical signs and symptoms in participants who present with and without symptoms of dry eye.
- Chapter 6: To compare tear osmolality and ferning patterns in participants with and without dry eye symptoms.

- Chapter 7: To investigate the potential relationship between subjective symptoms and clinical signs with tear film lipocalin and lysozyme concentrations in participants with and without dry eye symptoms.
- Chapter 8: To optimize a technique for the isolation of total RNA (ribo nucleic acid) and total protein derived from conjunctival epithelial cells collected via conjunctival impression cytology (CIC).
- Chapter 9: To quantify the expression of MUC1 (mucin1) and MUC16 (mucin16) mRNA and protein and to investigate the potential relationship between mucin expression and tear film breakup time in a group of participants with and without dry eye symptoms.

Methods

- Chapter 4: Participants were categorized as being symptomatic or asymptomatic of dry eye based on their response to the OSDI questionnaire. These results were then compared to the DEQ, which has questions related to the frequency of ocular surface symptoms and their diurnal intensity.
- Chapter 5: Non invasive tear breakup time (NITBUT) was evaluated using the ALCON Eyemap®. Tear volume was assessed using the Phenol Red Thread (PRT) test and bulbar conjunctival hyperemia was measured using objective (SpectraScan PR650© Spectrophotometer) and subjective (slit lamp) methods.
- Chapter 6: Tears were collected via capillary tube. A freezing point depression osmometer was used to measure the osmolality of the tear film. The tear ferning test was performed and evaluated for the quality of ferning, based on the Rolando grading system.

- Chapter 7: Tears were collected via capillary tube and an eye wash method. Tear lysozyme and lipocalin concentrations were determined via Western blotting.
- Chapter 8: CIC was collected using either Millipore (MP) or Poly Ether Sulfone (PES) membranes. RNA and protein isolation was performed using two different RNA isolation techniques. Two methods of protein isolation from CIC discs were evaluated. RT-PCR of mRNA for MUC1 and western blotting of lipoxygenase type 2 protein (LOX2) was performed to confirm the collection of intact RNA and total protein respectively.
- Chapter 9: Tears were collected via capillary tube and an eye wash method. CIC was collected using MP membrane. Expression of MUC1 and MUC16 mRNA was assessed via real time PCR. Expression of both membrane-bound and soluble MUC1 and MUC16 were quantified via Western blotting.

Results

- Chapter 4: The OSDI total score and sub scores for the Non Dry Eye (NDE) and Dry Eye (DE) groups were significantly different (NDE =7.43 ± 7.71 vs DE = 24.87 ± 13.89; $p<0.001$). The DEQ scores showed that the DE group exhibited a higher frequency and intensity of symptoms than the NDE group, which worsened as the day progressed ($p<0.001$).
- Chapter 5: The DE group exhibited a significantly shorter NITBUT (5.3 ± 1.7 vs 7.0 ± 2.7 secs; p=0.0012). Tear volume was significantly lower for the DE group (19.3 ± 5.1mm vs. 16.3 ± 5.6mm; p=0.031). Bulbar hyperemia was significantly higher in the DE

group for both objective (u' = 0.285 ± 0.006 vs. 0.282 ± 0.006; p=0.005) and subjective techniques (48.4 ± 10.0 vs 40.6 ± 10.4; p=0.0011).

- Chapter 6: Osmolality values in DE individuals were significantly higher than the NDE (328.1 ± 20.8 vs. 315.1 ± 11.3 mOsm/kg; p = 0.02). There was a significant difference between the DE and NDE participants for the ferning patterns (p = 0.019). No significant correlation between tear osmolality and tear ferning was noted (DE: r = 0.12; p>0.05, NDE: r = -0.17; p>0.05).
- Chapter 7: No difference in tear lysozyme or lipocalin concentration was found between DE and NDE groups, irrespective of tear collection method. Method of collection significantly influenced absolute concentrations (p<0.008).
- Chapter 8: There was no significant difference between the two procedures used to isolate RNA and protein from CIC membranes (p>0.05). Total RNA yield was greater with the MP membrane. The mean yield of protein extracted from MP membrane using the two protein isolation techniques also did not show a significant difference.
- Chapter 9: No difference was found in the expression of either MUC1 or MUC16 protein or mRNA expression between symptomatic DE and NDE (p>0.05). Weak correlations were found between the NITBUT values compared with either soluble or membrane bound MUC1 and MUC16 expression.

Conclusions

- Chapter 4: Questionnaires are useful tools to symptomatically divide participants into dry eyed and non dry eyed candidates. However, the questionnaire used to categorise patients can impact on the outcome variables determined.
- Chapter 5: Post-menopausal women with dry eye symptoms demonstrate shorter NITBUT, lower tear volume and increased bulbar conjunctival hyperemia than those who have no symptoms.
- Chapter 6: Tear osmolality in DE is higher than in NDE. There is a tendency towards less ferning in persons over 50 years of age, regardless of their symptoms.
- Chapter 7: Comparison of clinical data with lipocalin and lysozyme concentrations failed to reveal statistically significant correlations. The concentration of either protein was not associated with tear stability or secretion.
- Chapter 8: The total RNA yield was greater with the MP membrane. RNeasy™ Mini (RN) (Qiagen) method is recommended due to enhanced speed as well as on-column isolation and DNase digestion capabilities. CIC with MP membranes followed by immediate freezing and then extraction and processing facilitates the collection of total protein from human conjunctival cells.
- Chapter 9: No difference was found in the expression of either MUC1 or MUC16 protein or mRNA expression between symptomatic PMW and asymptomatic controls.

In this PhD project, over 125 participants were screened, and a total of 86 postmenopausal participants were enrolled. The table below (table A-1) represents the total

number of participants (out of the 86 enrolled participants) who participated in the following clinical and analytical studies. For the purpose of these studies, "postmenopausal" was defined as no menses for at least one year, not associated with hysterectomy. Participants were over 50 years of age.

Table A-1: Number of participants involved in the studies

Study	Number of post-menopausal women who completed the study
Assessment of Ocular Surface Dryness Using Dry Eye Questionnaires in Postmenopausal Females	82
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Tear Osmolality and Ferning Patterns in Postmenopausal Women	37
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Dedication

To my parents and brother.

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List of Symbols and Abbreviations

μg	micrograms
μl	microlitre
AU	arbitrary units
bp	base pair
BSA	bovine serum albumin
CCLR	Centre for Contact Lens Research
CCLRU	Cornea and Contact Lens Research Unit
cDNA	complimentary deoxyribonucleic acid
CIC	conjunctival impression cytology
CsCl	Cesium chloride
ddH2O	double distilled water
DE	dry eye
DEQ	dry eye questionnaire
DES	dry eye syndrome
DEWS	dry eye workshop
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	Dithiothreitol
EB	extraction buffer
EDTA	Ethylenediamine tetra acetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GLB	gel loading buffer
H&E	hematoxylin and eosin stain
HRT	hormone replacement therapy
KCl	Potassium chloride
KCS	keratoconjunctivitis sicca
KDa	KiloDalton
l	wavelength
L	Litre
LOX2	lipoxygenase type 2
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
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
NITBUT	non-invasive tear break up time

nm	nanometre
NS	not significant
OD	optical density
OSDI	ocular surface disease index
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
PM1	postmenopausal women study 1
PM2	postmenopausal women study 2
PMW	postmenopausal women
PRT	phenol red thread test
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
R2	correlation coefficient
rcf	relative centrifugal force
Rn	normalized reporter dye fluorescence
RN	Rneasy™ Mini kit
RNA	ribo nucleic 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
SIDEQ	subjective evaluation of symptoms of dryness questionnaire
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.05% Tween® 20
TFT	tear ferning test
TOS	total score
Tris	Tris (hydroxymethyl) aminomethane
TZ	TRIzol™
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume
WB	Western blotting
β	Beta
° C	degree Celsius

1 Introduction

Dry eye syndrome is one of the most frequently encountered ocular conditions. Dry eye is a common source of discomfort that can seriously affect a patient's quality of life, especially in the elderly population.[1, 2] Epidemiological studies have found that women are more likely to report dry eye symptoms than men[3, 4] and it is a condition that has a multifactorial etiology, which, in most cases, is always chronic and progressive.[5] There are many subjective and objective methods to test for dry eye. However, to date, there is no single test or combination of tests known to conclusively diagnose this disease.

A key aspect of dry eye that remains a major problem is the lack of association between the symptoms and signs of dry eye[6-14] and the poor test reproducibility of objective tests,[15] making it difficult to assess disease progression or the impact of treatments on symptoms. Currently, the major management for those patients with dry eye disease consists of palliative regimens such as lubricating drops, which target symptoms alone, with no treatment modality available that truly "treats" the underlying cause of the disease. The necessity for characterizing and understanding the underlying biomarkers in the tears and ocular surface cells that are involved in the disease process may be beneficial in targeting towards treatment strategies.

Many factors are involved in the development of dry eye. Of these, age, gender and hormonal effects have captured much attention over the past years. However, to-date, no specific study has been conducted on a group of postmenopausal women (who are not on hormone replacement therapy) to examine commonly reported dry eye symptoms, in

conjunction with a systematic analysis of the presence of various biomarkers in their tears and conjunctival epithelial cells.

Hence the focus of this thesis was as follows:

- Assessment of ocular surface dryness using dry eye questionnaires, including the Allergan Ocular Surface Disease Index (OSDI) and Indiana Dry Eye Questionnaire (DEQ) in postmenopausal women (chapter 4).
- Assessment of clinical signs (Non invasive tear break up time, Phenol red thread test, subjective and objective bulbar conjunctival redness) and symptoms (OSDI scores) in postmenopausal women with symptoms of dry eye (chapter 5).
- Assessment of tear osmolality and ferning patterns in postmenopausal women with symptoms of dry eye (chapter 6).
- Assessment of tear lipocalin and lysozyme concentrations (capillary and eye wash tear collection) in postmenopausal women with symptoms of dry eye (chapter 7).
- Optimization of a method for the isolation of total RNA and total protein from human conjunctival epithelial cells collected via impression cytology (Chapter 8).
- Evaluation of the expression of MUC1 and MUC16 mRNA and protein levels in tears and conjunctival epithelial cells collected from postmenopausal women with symptoms of dry eye (chapter 9).

The remainder of the thesis is composed of a literature review of dry eye (Chapter 2), a methods chapter introducing the subjects, clinical and analytical methods used in the study (Chapter 3), and general discussion and conclusions & recommendations for future work (Chapters 10 and 11).

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2 Literature Review on Dry Eye

2.1 Dry eye definition and classification

The National Eye Institute/Industry workshop in 1995 defined dry eye as *"a disorder of the tear film due to deficiency or excessive tear evaporation which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort"*.[1] This report divided dry eye primarily in two groups, namely tear deficient dry eye and evaporative dry eye (Figure 2-1). In light of extensive research in the area of dry eye over the past 10 years, and an improved understanding of the pathophysiological mechanisms, symptoms, tear hyperosmolality and effects of dry eye on vision, a new definition has been proposed. The most recent definition proposed by the International Dry Eye Workshop conducted in 2007 defines dry eye as a *"multifactorial ocular surface disease diagnosed by symptoms of discomfort and signs of visual disturbance, tear film instability and ocular surface damage, accompanied by increased osmolarity of the tear film and ocular surface inflammation."*[2] The terms "aqueous-deficient dry eye" and "evaporative dry eye" were removed from the earlier definition, but are still retained in the etiopathogenic classification of dry eye and reflects an improved understanding of dry eye (Figure 2-2).

Dry eye is believed to be a disturbance of the ocular surface functional unit. The ocular surface functional unit comprises the ocular surface (cornea, conjunctiva, meibomian glands), lacrimal glands, lids and the sensory and motor nerves that connect them.[3] (Figure 2-3) The overall function of the lacrimal gland functional unit is to

preserve the integrity of the tear film, the transparency of the cornea, and the quality of the image projected onto the retina.[3-5]

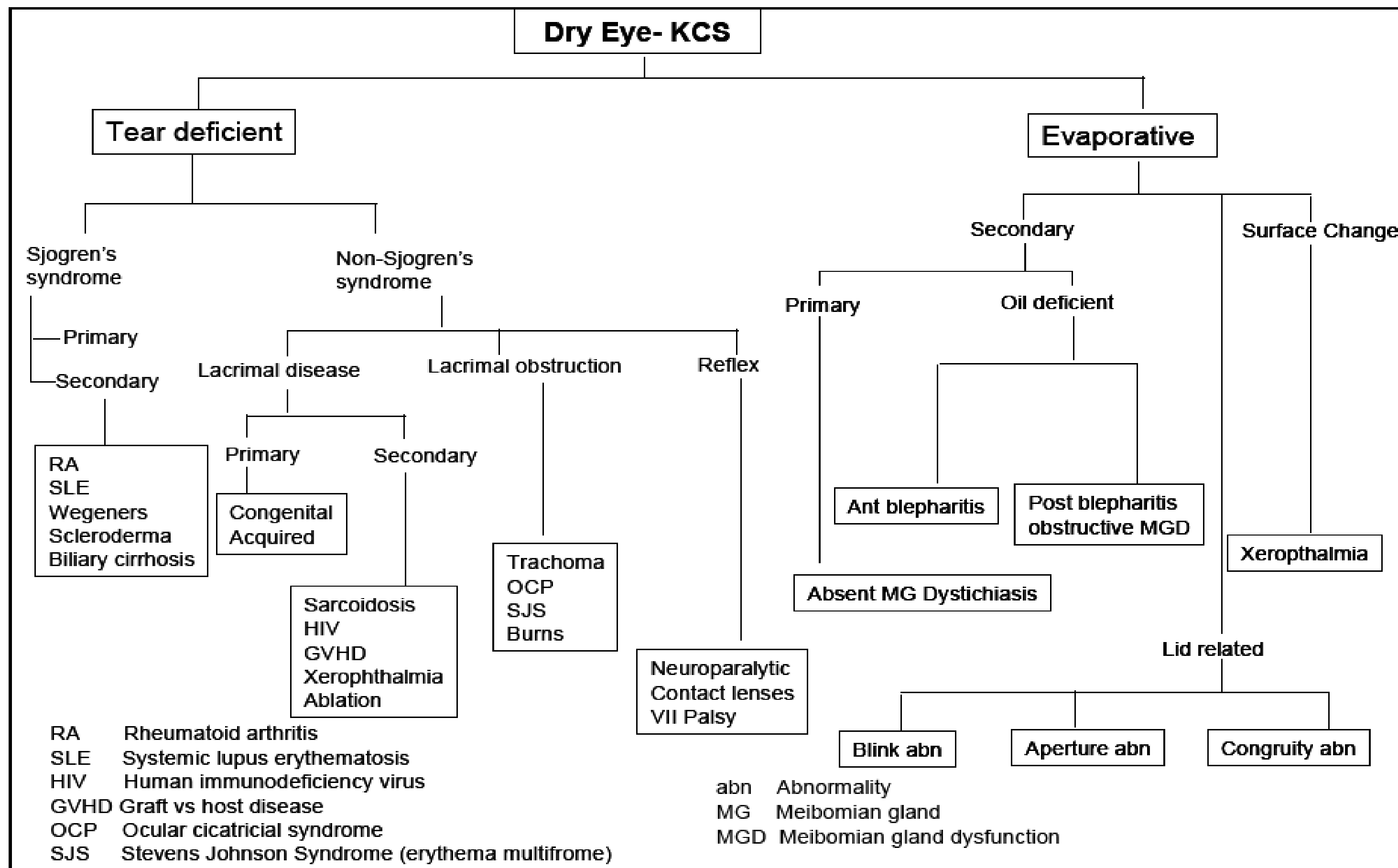

Figure 2-1: NEI/Industry workshop classification of dry eye

The two main types and causative factors of dry eye are shown in the flowchart.[1] Reprinted from CLAO Journal. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes.;1995;21:221-32, with permission from Lippincott Williams & Wilkins, Inc.

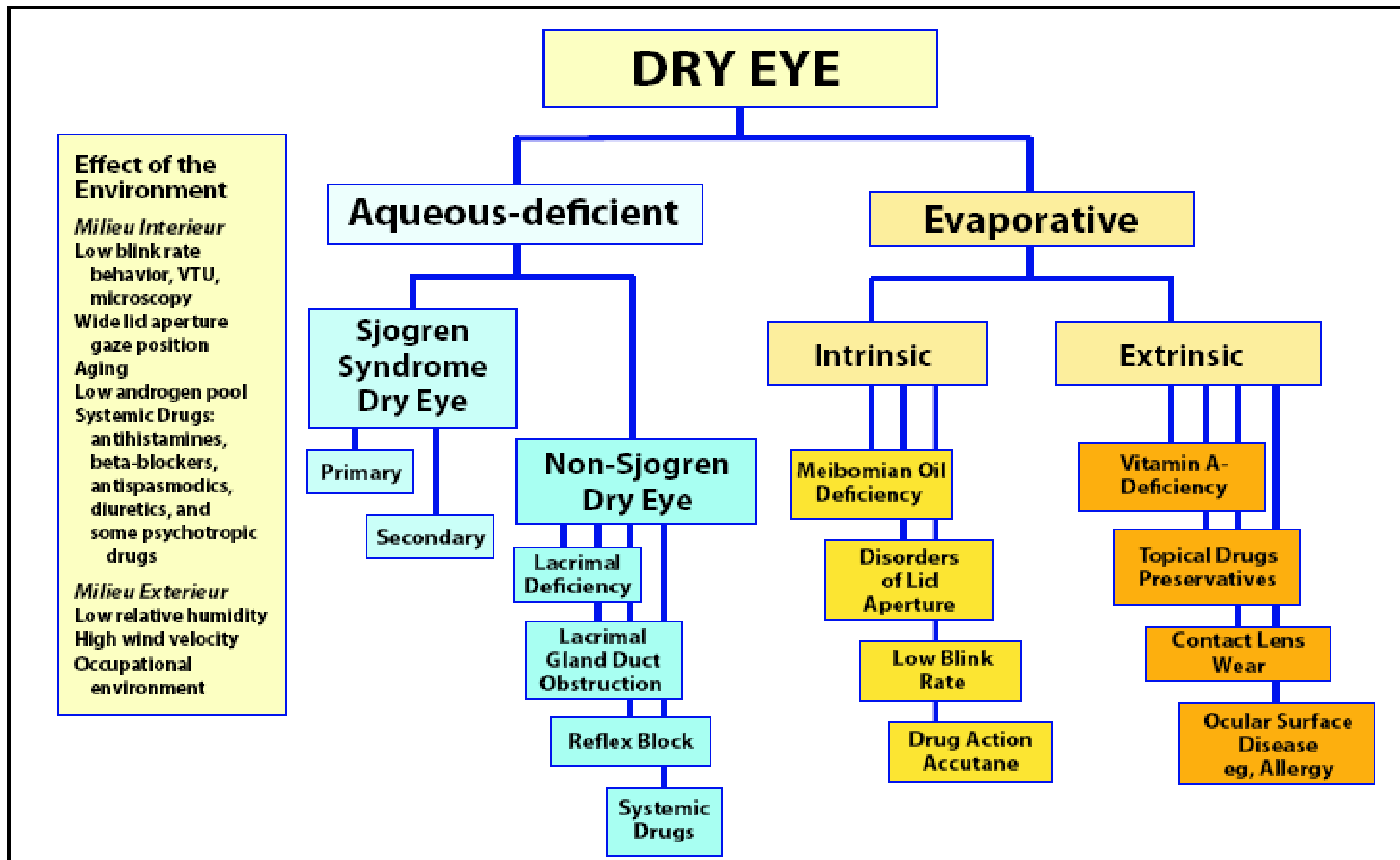

Figure 2-2: Etiological causes of dry eye

The left hand box illustrates the influence of environment on the risk of an individual to develop dry eye.[2] DEWS definition and classification of dry eye.2 Reprinted from The Ocular Surface Journal. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). Ocul Surf 2007;5:75-92, with permission from Elsevier.

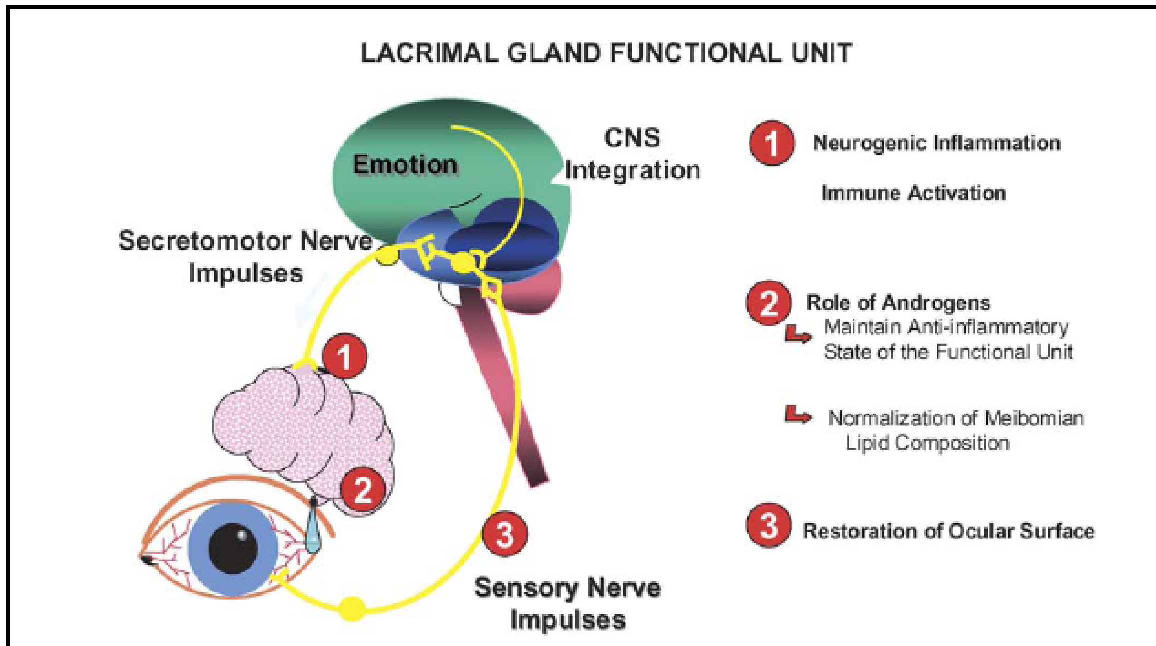

Figure 2-3: Schematic of lacrimal functional unit

Reprinted from Experimental Eye Research, Stern ME, Gao J, Siemasko KF, Beuerman RW, Pflugfelder SC. The role of the lacrimal functional unit in the pathophysiology of dry eye.[5] Exp Eye Res 2004;78:409-16, with permission from Elsevier.

2.2 Classification of dry eye

The current classification system shown in Figure 2-2 is an updated version of that presented in the NEI/Industry workshop report. Aqueous-deficient dry eye has two major groupings, Sjogren's syndrome dry eye and non-Sjogren's syndrome dry eye. Evaporative dry eye may be either intrinsic or extrinsic. Intrinsic refers to situations where the regulation of evaporative loss from the tear film is directly affected and extrinsic evaporative dry eye includes those etiologies that increase evaporation by their pathological effects on the ocular surface.

2.2.1 Aqueous tear deficient dry eye (ADDE)

Tear deficient dry eye is caused by either a failure in transporting lacrimal fluid to the conjunctival sac (resulting in a decreased amount of tears in the conjunctival sac) or a disorder in lacrimal gland function. Due to the reduced tear aqueous pool, the tear film osmolality is increased. Tear film hyperosmolality causes hyperosmolality of the ocular surface cells and hence triggers a cascade of inflammatory events, involving different signaling pathways and resulting in the generation of numerous inflammatory cytokines.[6,7] These include interleukin (IL)1 alpha, IL1 beta, tumor necrosis factor (TNF) alpha and various matrix metalloproteinases (eg MMP-9). Inflammatory mediators are generated from the lacrimal gland itself in cases of lacrimal gland dysfunction and inflammation (which can be found in tears) or from the cornea and conjunctiva. ADDE has been further classed into Sjogren's syndrome, an autoimmune disorder affecting the lacrimal and salivary glands, and non-Sjogren's syndrome tear deficient dry eye, which includes other causes of aqueous deficiency.

2.2.1.1 Sjogren's syndrome dry eye (SSDE)

Sjogren's syndrome is a chronic autoimmune disorder of the exocrine glands, with associated lymphocytic infiltration of the affected glands. Sjogren's syndrome is the most under-diagnosed autoimmune disease,[8, 9] which affects about 0.3% of the population [10] and occurs mainly in women (>90%).[11, 12] The exocrinopathy can be either a primary disorder (primary Sjogren's syndrome) or it can be associated with other connective tissue diseases (secondary Sjogren's syndrome), such as rheumatoid arthritis, systemic lupus erythematosis or systemic sclerosis.[13]

The etiology of Sjogren's syndrome is not very well understood, however viral, neural, genetic and environmental factors are all thought to contribute to the condition.[14,15] Sjogren's syndrome is a T-cell driven autoimmune disease and can be characterized by focal lymphocytic infiltration of the lacrimal and salivary glands,[16] presence of circulating autoantibodies produced by B cells[17] and increased expression of several proinflammatory cytokines.[11] These inflammatory cells impair the functions of the lacrimal gland, causing tear deficient dry eye.[9] The sub-classifications of aqueous deficient dry eye are illustrated in Figure 2-2.

2.2.1.2 Non Sjogren's syndrome dry eye (NSSDE)

There is no associated systemic autoimmune feature involved in NSSDE. The reduction in tear secretion is caused by other factors, including primary lacrimal gland deficiencies that may be age related,[18] due to congenital alacrima (absence of lacrimal gland)[19, 20] or familial dysautonomia (a disorder of the autonomic nervous system which affects the development and survival of sensory, sympathetic and some parasympathetic neurons in the autonomic and sensory nervous system, resulting in various symptoms, including inability to produce tears).[21]

Lacrimal gland infiltration due to sarcoidosis,[22] lymphoma,[23] AIDS,[24] graft versus host disease,[25, 26] lacrimal gland excision[27, 28] or denervation[29] may cause secondary lacrimal gland deficiency that is associated with NSSDE. Obstruction of the lacrimal gland and accessory lacrimal gland ducts can lead to ADDE. Conditions which cause scarring of the cornea such as chemical burns,[30] cicatricial and mucous membrane phemphigoid, erythema multiforme and trachoma can all produce lacrimal obstruction, resulting in tear deficient dry eye.[13] A reduction in the sensory impulses from the ocular

surface can cause dry eye by either decreasing reflex-induced lacrimal secretion or by reducing the blink rate, which leads to excessive evaporation of tears.[31] Corneal sensitivity reduction due to contact lens wear or post LASIK surgery can possibly cause symptoms of dry eye.[32, 33] Large population based studies have also identified diabetes mellitus as a risk factor for dry eye disease.[34-36]

2.2.2 Evaporative dry eye

In patients with evaporative dry eye, there is increased tear evaporation from the ocular surface, with normal lacrimal gland tear production and flow. However, occasionally a combination of both aqueous deficiency and increased evaporation may contribute to the dry eye condition. Evaporative dry eye can be due to either intrinsic disease factors (affecting lid structures or dynamics) or it may be extrinsic, where ocular surface disease occurs due to some extrinsic factor. Intrinsic factors are further classified into oil deficient (due to meibomian gland disorders), lid related, blink rate related and surface change. Extrinsic factors include ocular surface disorders caused due to vitamin A deficiency, use of topical drugs and effects due to their preservatives, contact lens wear and ocular allergies. All of these causative factors for evaporative dry eye are illustrated in Figure 2-2. [2]

2.2.2.1 Effect of the environment

Environmental factors can play an important role in dry eye. As quoted in the DEWS workshop report, the term "environment" is used to describe psychological variations between individuals, (milieu interieur) as well as the conditions that they encounter (milieu exterieur). Low relative humidity conditions and the general occupational environment all contribute towards the conditions that an individual

encounters. Factors such as low blink rate behavior[37] (which increases the evaporative loss between blinks),[38] ageing, androgen levels, and systemic drugs can contribute towards psychological variations between individuals.

2.2.2.2 Environmental factors (milieu interieur)

People using anti-histamines, antispasmodics, diuretics or steroids have a higher incidence of dry eye, but people using angiotensin converting enzyme (ACE) inhibitors have a lower incidence. Although arthritis and thyroid disease are not associated with the incidence of dry eye, arthritis is more likely to develop in people with dry eye, suggesting that dry eye may precede the development of arthritis.[36] The evaporation of tears increases with increasing palpebral aperture width, and is hence the most in upgaze.[38] Ageing[18] and androgen deficiency[39] may be triggering factors in causing dry eye.

2.2.2.3 Environmental factors (milieu exterieur)

An indoor working environment, low blink rates, particularly in those workers using visual display terminals (VDT) in air-conditioned workplaces, has been identified as the main environmental causative factor of dry eye.[40] The reduction in blink rate due to VDT use results in increased dry eye symptoms.[41] Environmental irritants associated with ‘sick building syndrome’ have been reported to cause ocular irritation and tear film instability in office workers.[42, 43] It has also been shown that low humidity levels in planes during long-haul flights and changes in blink pattern may cause dry eye symptoms.[44] Other activities associated with decreased blinking and increases in palpebral aperture width have been reported to carry a risk for the development of dry eye symptoms.

2.2.3 Ageing and dry eye

Ageing, a normal biological process occurring on a cellular and tissue level, may affect the tear film, with the ageing process leading to decreased function. Dry eye is more common in older patients[45] and recent epidemiological studies have consistently shown that the prevalence of dry eye symptoms increases with age,[35, 46-50] with the prevalence reaching a plateau after the mid 80's.[51] There is a related reduction in tear production[18] and increased meibomian gland dysfunction[52] with age. There is a decrease in reflex secretion of tears with age, particularly after 40 years of age.[53] This reduction in reflex tear production is attributed to decreased corneal sensitivity with age.[54] Earlier studies have shown a decrease in basal tear secretion with aging.[55, 56] However, later studies of tear turnover by modern fluorophotometric methods do not show an age-related variation in tear production.[57] The rate of tear film evaporation has been shown to be constant throughout life,[58] but has been reported to increase with age in another publication.[18] Ageing can also associated with decreased tear volume and flow, increased osmolality,[18, 59] decreased tear film stability[60] and alteration in the composition of tear film lipids.[61]

Clinical observations suggest that dry eye occurs more in women, particularly after menopause. However, women seek eye-care more frequently than men,[51] potentially skewing this observation. Recent epidemiological studies support the fact that there is a higher prevalence of dry eye among women.[62] A higher proportion of females have aqueous tear deficiency than men[10] and an increased prevalence of all categories of treated dry eye occurs in women, compared with men.[63]

Sex hormones are believed to play a significant role in causing dry eye.[39] A reduction in sex hormones, such as androgens, occurs in both males and females with increasing age.[39, 61] The hormonal changes that accompany menopause can also play an important role in the production of dry eye symptoms.[8, 64, 65] Estrogen therapy, which is commonly taken by postmenopausal women, are also thought to be a risk factor for the development of dry eye.[50, 66] A significant decrease in androgen levels is also associated with meibomian gland dysfunction.[67]

2.2.3.1 Postmenopausal women and hormone replacement therapy

Large epidemiological studies have clearly shown that the prevalence of dry eye is greater among women than in men, especially women aged 50 and over.[50] More women complain of dry eye symptoms and show clinical signs than men.[62]

Menopause may play an important role in the development of dry eye.[8, 64] The impact of hormones on the incidence and course of dry eye, especially in postmenopausal women has been noted.[64] Researchers have demonstrated the presence of α-type and β-type estrogen receptors in the epithelia of several ocular tissues and have suggested that sex steroid hormones may play a role in the development of certain ocular diseases.[68, 69] Evidence demonstrates that the meibomian glands of the lids contain androgen, estrogen and progesterone receptor mRNA and protein within the acinar epithelial cells, and that these respond to androgen precursor by increasing their production and release of lipids.[70, 71]

Studies conducted by Sullivan et al[72-74] and others[75, 76] have clearly demonstrated that the meibomian gland is an androgen target organ and that androgens impact meibomian gland function, regulate the quality and quantity of lipid produced and

promote the formation of the tear film lipid layer. Hence, any deficiency associated with androgens can cause meibomian gland dysfunction and an increase in the signs and symptoms of dry eye.[72-74] The decline in the levels of total androgen with ageing in both sexes,[77, 78] particularly in females upon the onset of menopause, coincides with the increased appearance of meibomian gland dysfunction and ageing.[79]

Hormone replacement therapy (HRT) refers to the administration of estrogens and progesterones to post-menopausal women and is commonly used to reduce signs and symptoms of post menopause.[80] HRT is an established method for alleviating menopausal complaints [80] and had been previously thought to be beneficial for the cardiovascular system. HRT has also been shown to improve bone density and lipid metabolism.[80] However, various deleterious effects following HRT administration,[81-83] including an increased risk of cancer, have been reported.[82] There are confounding results in the literature regarding the risks and benefits of HRT on dry eye.[66, 84] Schaumberg et al[62] suggested that women who had HRT with estrogen and estrogen plus medroxyprogesterone acetate were at an increased risk of dry eye syndrome. Other studies have shown the opposite effect.[85, 86]

HRT which has estrogen, progesterone and androgen activity with high tissue specificity has shown an improvement in the values of Schirmer's test and tear break up time (TBUT).[87] Schaumberg et al[66] also suggested estrogen therapy alone caused ocular dryness and that a balance between androgens and estrogens are important in determining the risk of dry eye syndrome. It was hence suggested that addition of progesterone along with estrogen therapy may be beneficial.[66]

2.3 Prevalence of dry eye

Reports suggest that a large number of patients with dry eye problems visit eye-care professionals, with one in every four patient visits to an ophthalmologist being related to complaints of dry eye[45] and 17% of visits to eye centers being due to dry eye.[40] About one-third of the general population have occasional symptoms of dry eye.[88] Table 2-1 lists some of the major epidemiological studies that were undertaken in the past 10 years and these results indicate that the prevalence of dry eye ranges from 3.5% to approximately 55%. The criteria used to confirm a "dry eye diagnosis", as shown in Table 2-1, differs widely between studies, resulting in great difficultly when attempting to compare results across studies. The majority of studies have used symptoms as diagnostic criteria, as dry eye is largely a symptom-driven disease. In addition to differences in dry eye definition, other factors such as variability in sampling techniques, age groups, geographical variations, measurement techniques, cut-off values for these measures and racial differences all contribute to differences in the results obtained.[89] Due to the lack of a single diagnostic test or a combination of tests to effectively diagnose dry eye, many studies have reported a lack of correlation between symptoms and signs of the disease.[59, 90, 91]

Studies undertaken in the US estimate that over 3.2 million women and 1.6 million men aged over 50 years suffer from moderate to severe dry eye.[50, 92, 93] One of the reports [46] suggests that diagnosing dry eye by symptoms alone is acceptable because dry eye rarely progresses to the stage of causing ocular damage without symptoms being present. However, more recent reports suggests that symptoms alone are inadequate for

diagnosing dry eye because the same symptoms can be experienced by patients with a large range of ocular surface conditions and tear film disorders.[62,87]

Table 2-1: Epidemiological data from dry eye studies

Study	Number of participants and age	Diagnostic criteria	Prevalence
Blue mountain study [49] (2003)	1174 subjects (>50 years)	Questionnaire: • at least one symptom • moderate to severe symptoms • 3 or more symptoms	57.59% 16.6% 15.3%
Women's Health Study [50] (2003)	39,876 women (≥ 49yrs)	History of diagnosed dry eye or dry eye symptoms	7.8%
Physicians Health study I &II [92, 93] (1998 & 2002)	25, 655 (>50, 55yrs)	Severe symptoms of dryness and irritation and /or physicians diagnosis	
Canadian Optometric Practices (CANDEES) [88] (1997)	13,517 subjects	Questionnaires based on symptoms and self diagnosis	28.7%
Beaver Dam Study [35] (2000)	3722 subjects (48-91 years)	Based on symptoms	14.4%
The Shihpai Eye Study [62] (2003)	2038 subjects (≥ 65 years)	1 or more dry eye symptoms	33.7%
Indonesian Population study [48] (2002)	1058 subjects (>21 years)	Questionnaire: 1 or more of 6 symptoms present often or all of the time	27.5%
Salisbury Eye Evaluation study [94-96] (1997, 1999, 2000)	2420 subjects (>65-84 years)	• Questionnaire • Schirmer test ≤ 5 mm or Rose Bengal ≥ 5	14.5% 3.5%

Japanese Eye Centres [40] (1995)	2127 patients 10-89 years	All 3 criteria had to be positive: • More than one chronic symptoms • Rose Bengal score ≥ 4 or Fluorescein score ≤ 1 • TBUT ≤ 5 sec or Schirmer test with anasthesia • ≤ 5mm or Cotton thread test ≤ 10mm or tear fluid dilution test ≤ X4	17%
Melbourne Visual Impairment Study [47] (1998)	926 subjects 40-97 years	• Rose Bengal > 3 • Schirmer Test < 8 mm in 5 mins • Tear Break Up Time < 8 secs • 2 or more signs • Severe Symptoms not attributed to hay fever	10.8% 16.3% 8.6% 7.4% 5.5%
Copenhagen [97] (1997)	504 subjects 30-60 years	Two of the following tests had to be positive: • Schirmer-I test ≤ 10mm/5min • TBUT ≤10 sec • Rose Bengal Score ≥ 4 on 0-9 scale	11%

2.4 Causative mechanisms of dry eye

Recent evidence has shown that certain mechanisms can play an important role in initiating or exaggerating dry eye. They are thought to be related to tear film stability and tear hyperosmolality.

2.4.1 Tear film instability

The stability of the tear film is dependant on the quantity and quality of various components of the tear film, including tear viscosity, surface tension, meniscus radius, and initial and final film thicknesses.[98] When the tear film ruptures or tear break-up appears, it may produce tear film hyperosmolality and local drying of the exposed surface, leading to the excitation of inflammatory cell markers at the ocular surface, epithelial damage and cell death by apoptosis. This may cause a disturbance of the glycocalyx and goblet cell mucins.[2] These various disturbances exacerbate tear film instability, triggering a vicious cycle of events, graphically shown in Figure 2-4.

Various factors contribute towards the destabilization of the tear film.[99-101] Disturbances of the lipid layer result in increased evaporation of the tear film. Holly[99] proposed that the rapid break-up of tears could be due to the contamination of the mucin layer by inward movement of the superficial lipid, creating small hydrophobic areas which do not adequately support the aqueous phase. Liotet et al[100] suggested that the inability of the corneal epithelial cells to manufacture a glycocalyx may result in insufficient sites for mucous layer attachment, and this may be a major factor in determining tear stability.[100]

Van der Waals dispersion forces (attractive or repulsive forces between molecules) within the mucous layer may also cause disruption of the tear film.[101] Tear drainage due to gravity or a rising film height reaching the effective range of the dewetting intermolecular forces may also be responsible for the breakup of the tear film.[98, 102] These factors independently would be inadequate to cause tear film break-up,

however a combination of them will likely be adequate to cause disruption of the tear film.

2.4.2 Tear hyperosmolality

Tear hyperosmolality is thought to be one of the core mechanisms causing inflammation of the ocular surface (Figure 2-4) and is considered a central mechanism causing ocular surface damage and symptoms of dry eye. An increase in tear film osmolality is due to evaporation of tears from the ocular surface and/or a decrease in the production of tears from the lacrimal glands, or a combination of both events.

The terms osmolality and osmolarity are unfortunately used interchangeably and the terminology associated with calculated and measured osmotic activity is not consistent in the literature. Osmotic concentration determinations are typically expressed as either milliosmoles/kilogram (mOsm/kg) of solvent, referred to as “osmolality”, or milliosmoles/liter (mOsm/L) of solution, referred to as osmolarity. When the osmotic concentration is derived by an osmometer, in laboratories that use a method such as freezing point depression of water (or the vapor pressure technique), the concentration is expressed in terms of solvent and is appropriately referred to as “osmolality”.[103, 104] If it is expressed in terms of solution, the term osmolarity is appropriate.[92] The osmolarity of a solution is the total concentration of dissolved particles in a solution, irrespective of their size, density, molecular weight or electric charges.[105]

Tear osmolarity increases in dry eye, indicating an increased concentration of dissolved particles in the tear film volume. Inflammatory events are subsequently stimulated, due to hyperosmolarity and are thought to involve mitogen-activated protein kinase (MAP kinase) and nuclear factor - kB (NFkB) signaling pathways[6] and the

generation of inflammatory cytokines, primarily interlukin 1 α (IL 1 α), interlukin 1 β (IL 1 β); tumor necrosis factor α (TNF α) and matrix metalloproteinases (MMPs), which initiate inflammatory cells at the ocular surface.[8] Stern and Pflugfelder reported that the expression of IL 1 α and β, IL 6, IL 8, TGF β, and TNF α were increased in the conjunctival epithelia of patients with Sjogren's syndrome compared with controls.[106] Inflammatory events lead to apoptosis of epithelial cells, including goblet cells. Zhao et al[107] and Argueso et al[108] demonstrated reduced levels of MUC5AC in dry eye, and goblet cell loss is seen is all forms of dry eye. It has been hypothesized that a faster tear thinning rate is a risk factor for tear hyperosmolarity[2] and a wide variation of thinning rates[109] have been noticed. Normal individuals with fastest tear thinning rates experience increased higher tear osmolarity.[110]

Reduced aqueous tear flow, resulting from lacrimal failure, and/or increased evaporation from the tear film are the major causes of tear hyperosmolarity. Another potential reason is meibomian gland dysfunction (MGD), which leads to an unstable tear film lipid layer. Aging and the use of systemic drugs such as antihistamines and anti-muscarinic agents may induce reduction of aqueous flow. As previously described, environmental conditions of low humidity and high air flow can cause increased evaporative loss.

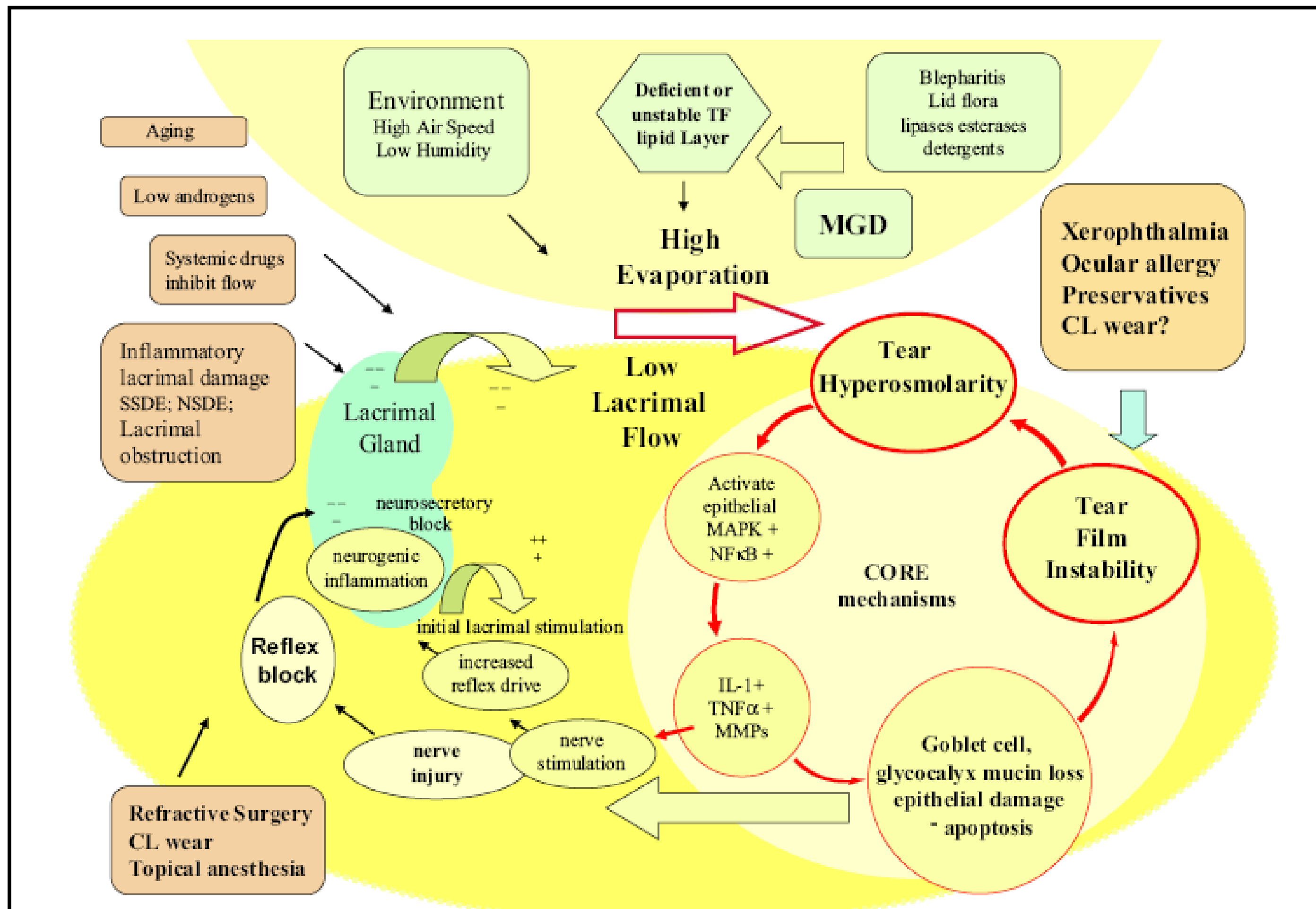

Figure 2-4: Causative mechanisms of dry eye

Reprinted from The Ocular Surface Journal. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007).[2] Ocul Surf 2007;5:75-92, with permission from Elsevier.

2.5 Risk factors for dry eye

Demographic factors including age, sex and many ocular, systemic, interventional & environmental factors can be associated or cause dry eye and can also worsen an already existing condition. A diet that is deficient in Omega 3 essential fatty acid or vitamin A, medications, smoking, alcohol, and menopausal status may contribute toward the risk factors for dry eye.

2.5.1 Age, gender and sex hormones

As explained in section 2.2.3 dry eye symptoms increase with age, especially in women and sex hormones are believed to play a role in causing dry eye.[39]

2.5.2 Contact lens wear

The prevalence of symptomatic dry eye in contact lens wearers occurs in 50-80% of wearers. Discontinuations and drop outs from lens wear are primarily due to symptoms of discomfort and dryness.[2, 111, 112]

2.5.3 Refractive surgery

Dry eye can occur as a complication of laser in-situ keratomileusis (LASIK) surgery.[31, 113-116] LASIK may result in lacrimal dysfunction and decreased goblet cell density, leading to reduced tear production and an unstable tear film.[114, 117]

2.5.4 Environmental factors and computer use

An indoor working environment can cause symptoms of ocular irritation,[118] particularly in those who use computers[119, 120] in air-conditioned workplaces, has been

identified as the main environmental causative factor of dry eye.[1] Low humidity, poor air quality, and pollution are other risks that may cause dry eye.[121, 122]

2.6 Impact of dry eye

Dry eye can interfere with day-to-day activities, including reading, computer use, night driving and watching television[123, 124] and can severely impair patient quality of life.[124, 125] Schiffman et al.[126] showed that the mean comorbidity-adjusted patient preference score values were similar for severe forms of dry eye and moderate to severe angina pectoris.[126] The economic impact of dry eye has to be assessed in terms of the cost of the visits to eye care professionals, treatment costs and the impact of the condition on the health and productivity of the patient.[127] A decade ago, 7-10 million Americans used artificial tears on prescription or as self-medication, at an annual cost of $100 million.[128] With growing knowledge of dry eye, this annual cost has significantly increased.

2.7 The tear film

Of all the components that constitute the lacrimal functional unit, the tear film is by far the most dynamic. It provides nutrients, a communication pathway, distributes regulatory factors and provides a pathway for cells to reach the epithelium. The tear film consists of a variety of different components, including electrolytes, salts, protein and peptides. Studies provide evidence that specific proteins or peptides in the tear film can be used as diagnostic biomarkers for dry eye, ocular surface diseases,[129] and even certain systemic disease states, such as diabetes mellitus.[130, 131]

The tear film has a number of specific functions:

1. It lubricates and nurtures the anterior tissues of the eye.
2. It provides a regular optical surface for the eye's optical system, by filling the irregularities of the corneal epithelium.
3. Oxygen dissolved in the tear film is the only source for normal aerobic metabolism of the corneal epithelium and stroma.
4. It is an integral part of the ocular surface defence mechanism.[132]
5. It also functions as a method to remove cellular debris and metabolic waste from the cornea and conjunctiva.

The integrity and normal functioning of the tear film is maintained by a complex physiological mechanism, which includes adequate production of the various components by different glands, stability of the various layers on the ocular surface and timely drainage through the lacrimal ducts. Any disruption in the physiology or stability of the tear film may result in dry eye.

In 1946, Wolff [133] presented a classic description of the tear film, in which it was composed of a three-layered structure, consisting of an anterior lipid layer, middle aqueous layer and deeper mucin layer (Figure 2-5).

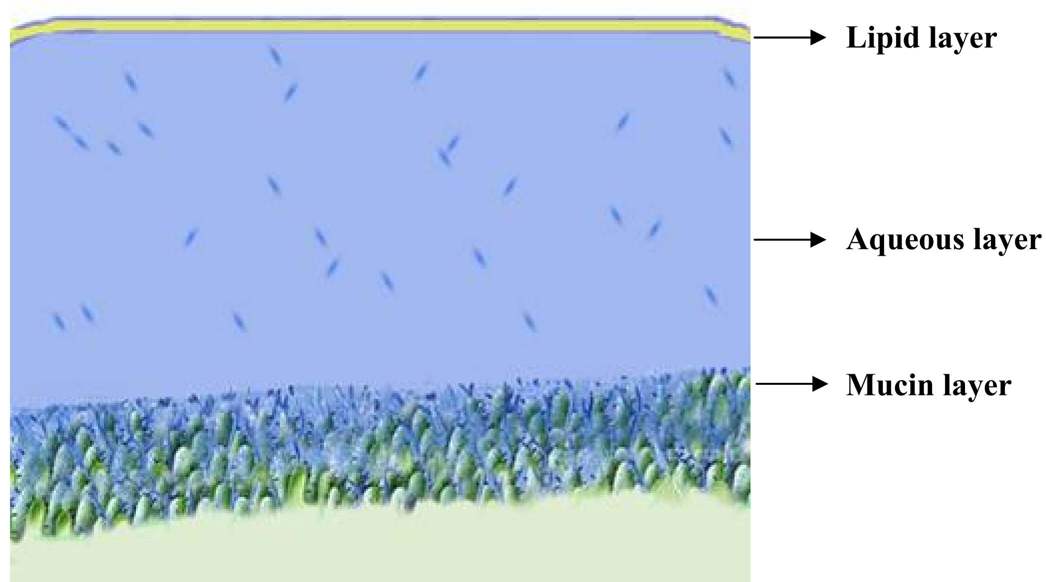

Figure 2-5: Wolff's three-layered structure, consisting of an anterior lipid layer, middle aqueous layer and deeper mucin layer

Image adapted from http://www.lea-test.fi/en/eyes/images/pict7b.jpg

Another model with six layers has also been proposed by Tiffany,[134] which included the original three layers proposed by Wolff, along with air-lipid, lipid-aqueous and aqueous-mucus interfaces. More recently it has been suggested that there are dissolved mucins in the aqueous layer, which decrease in concentration towards the lipid layer.[135] The most currently accepted current concept is that the tear film is a bilayered structure, consisting of an aqueous/mucinous phase, with an overlying superficial lipid phase (Figure 2-6).[136] However, despite the intervening 60 year period, the original 3 layered Wolff model is still accepted as being a valuable concept.[137]

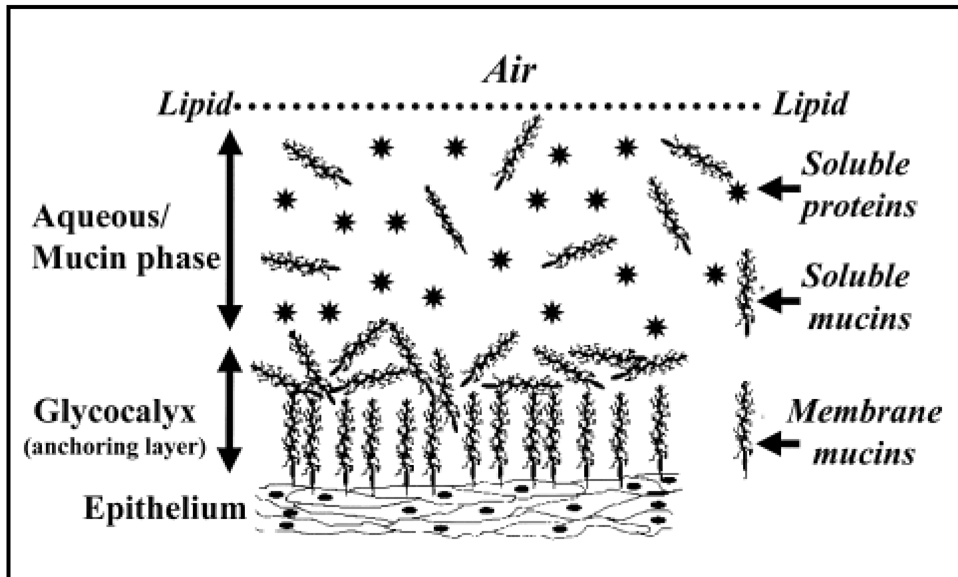

Figure 2-6: Proposed structure of precorneal tear film

The precorneal tear film consists of a superficial lipid layer, a middle aqueous/mucin phase that contains soluble mucins, aqueous fluid, electrolytes, and proteins that are secreted by the lacrimal glands, and ocular surface epithelium. This layer is anchored to the underlying superficial corneal epithelium by chemical attractions to the epithelial membrane mucins (glycocalyx). Reprinted from Cornea. The Diagnosis and Management of Dry Eye. A Twenty-five–Year Review. Cornea 2000, 19(5): 644–649, with permission from Lippincott Williams & Wilkins, Inc.

The thickness of the tear film is vitally important to understand, because the thickness determines the tear volume from which evaporation occurs when the eyes are open.[138] However, there is no agreement among researchers on the “true” thickness of the tear film. Various studies to investigate tear film thickness have been performed, using a variety of techniques, and these studies have quoted tear film thickness ranges from1.5µm to 45µm.[139-143] Prydal et al found the tear film thickness to be 34-45µm with coherence interferometry and confocal microscopy.[139, 140] More recently, King-Smith et al,[141] using wavelength- dependent interferometry, have reported the thickness of the tear film to be 1.5-4.7µm in normal subjects.

2.8 Layers of the tear film

2.8.1 The lipid layer of the tear film

Lipid forms the superficial/outermost layer of the tear film and is derived primarily from the meibomian glands in the eyelids.[144] It consists of an outer non-polar lipid layer and an inner polar layer.[145] The meibomian glands are richly innervated by both sympathetic and parasympathetic neurons.[146] Glands of Moll and lash follicle glands of Zeis contribute some lipid (Figure 2-7).[147] It is believed that androgen has an important role to play in the functioning of meibomian glands.[39, 73, 148] Several studies including clinical models and animal studies have shown that androgens increase the size, activity and lipid production in the meibomian glands.[72, 148] Tear lipocalin enhances the stability and spreading of the lipid layer, which decreases the surface tension of aqueous by forming complexes with polar lipids.[149]

2.8.1.1 Functions of the lipid layer

1. To provide an effective barrier and prevent evaporation.[150, 151]
2. To provide a surfactant layer that acts as an effective bridge between the non-polar lipid layer and the aqueous mucinous layer.[152]
3. To act as a lubricant to facilitate the movement of the eyelids during blinking.[153]
4. To maintain compression and expansion of the lipid film during blinks, to prevent tear overflow.[153]
5. To form a barrier for preventing contamination of the tear film.[154]
6. To provide a smooth surface for refraction of the incoming rays of light.[153, 155]

Defects and instability in the lipid layer can be responsible for tear breakup, with subsequent dry spots leading to dry eye.[156]

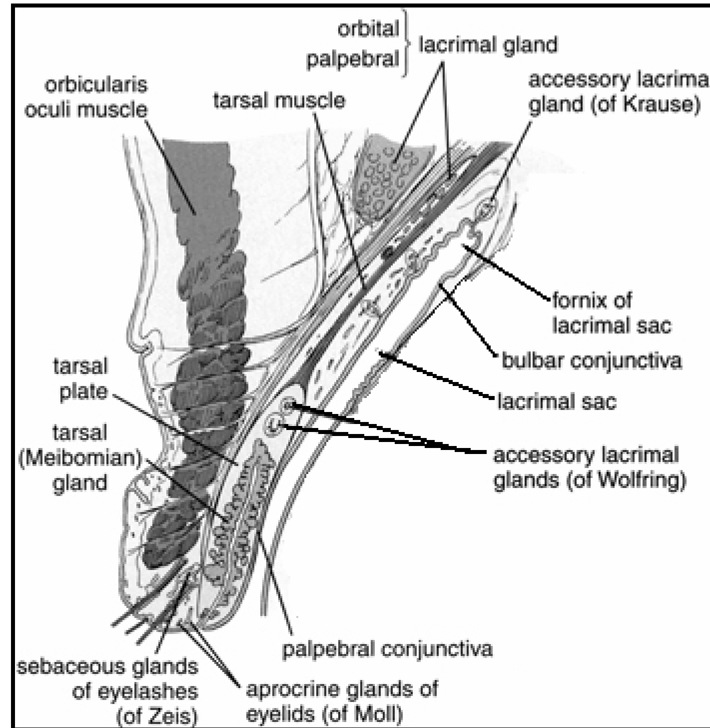

Figure 2-7: Representation of tear producing glands

Image adapted and modified from anatomy.ipui.edu/.../Eye04/palp.jpg

2.8.1.2 Lipid dynamics and drainage

With every down-phase of the blink, lipid is squeezed out of the meibomian glands and is compressed into the lid margins. On the up-phase of the blink, the lipid rapidly spreads upwards over the aqueous layer, suggesting that the lower eyelid reservoir is the major contributor to the spreading lipid.[137] Most of the excretion of the lipid layer occurs by bulk flow over the lid margin and onward to the neighboring skin and lashes. [137] Some of the lipid, mainly polar, mixes with the aqueous layer and is drained via the naso-lacrimal drainage pathway.[154]

2.8.1.3 Thickness of the lipid layer

Various interferometric techniques have been used in the past to study thickness of the lipd layer. Studies using specular microscopy have reported lipid thickness values of 100 nm.[157,158] Techniques such as specular reflectometry have shown the lipid thickness to be 13-70 nm.[159] Using photometric reflectometry, the lipid thickness was found to be 32-46 nm.[160]

2.8.1.4 Composition of the lipid layer

It is believed that the lipid layer has two distinct regions. There is a relatively thick outer layer, containing nonpolar lipids such as wax esters, sterol esters, hydrocarbons, and triglycerides; and a thin polar inner layer, predominantly consisting of phospholipids.[161] The polar phase of the lipid layer owes its surfactant properties which facilitate mixing with both aqueous and non-polar lipids It acts base for the more superficially located non-polar lipids.[145] The major classes of lipids are the wax monoesters and sterol esters, which comprise approximately three-quarters of the meibomian gland fluid.[145, 162, 163]

2.8.1.5 Physical properties of lipid

Although isolated meibomian lipids do not spread over saline, the lipid layer spreads well over the aqueous phase of the tear film.[158] The refractive index of the lipid layer varies with wavelength and temperature. Tiffany[154] estimated the refractive index of meibomian lipid to be 1.4766 at 589 nm and 35°C.

2.8.1.6 Methods to quantify lipids

Meibomian gland lipid can be obtained by squeezing the eyelid margin to express meibum,[164-166] by gently sucking meibum out of each glandular orifice[162] or collecting tear samples.[152, 167] These samples are then dissolved in an organic solvent and separated into their various lipid classes. One of the most widely used techniques to study lipids is analysis by high pressure liquid chromatography (HPLC) with or without mass spectroscopy (MS), or by gas chromatography followed by mass spectroscopy (GC-MS). In both the HPLC and GC methods, separation of lipids is followed by mass determination. Various other techniques are also available to study meibum samples. Approaches such as derivative gas chromatography electron ionization mass spectrometry (GC/EI-MC), positive chemical ionization, negative chemical ionization (NCI) of chloride adducts, electrospray tandem mass spectrometry (ES-MS/MS), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI) MS and APCI liquid chromatography (LC)/APCI-MS have been used for mass spectrometric analysis of acylglycerides, which are comprised of mono-, di-, and triglycerides.[168]

2.8.1.7 Tear lipids in dry eye

Analysis of lipid components has shown a considerable decrease in triglycerides and cholesterol, and monounsaturated fatty acids (specifically oleic acid), in patients with obstructive meibomian gland dysfunction, chronic blepharitis and viscous meibum.[165, 169] These changes in the lipid composition may be associated with tear film instability. Polar lipids may define the structural organization of the overlying non-polar lipids and loss of

these constituents are believed to result in decreased tear breakup time and increased aqueous tear evaporation.[170]

2.8.2 The aqueous layer

The aqueous layer of the tear film comprises the bulk of the tear film thickness. This intermediary watery phase of the tear film is approximately 6.5-7.5 μm thick[143] and is mainly produced by the main lacrimal gland, and also by the accessory glands of Krause and the accessory glands of Wolfring (Figure 2-7). It contains electrolytes, protein enzymes and metabolites.

2.8.2.1 Aqueous production

2.8.2.1.1 The lacrimal gland

The lacrimal gland, the main secretor of the aqueous phase of the tear film, is located in the upper outer part of the orbit in a shallow depression in the frontal bone and is divided into larger orbital and smaller palpebral lobes. The gland is a multi-lobed, tubulo-acinar structure with ducts that terminate at the surface of the eye in the lateral portion of the superior fornix.[171]

2.8.2.1.2 Accessory lacrimal glands

The accessory glands comprise the glands of Krause and Wolfring. There are more than 20 accessory glands of Krause in the upper conjunctival fornix and about 6-8 in the lower conjunctival fornix. These glands are located in the substantia propria of the conjunctiva. Glands of Wolfring are found in the upper border of the tarsal plate.[171]

2.8.2.2 Components of the aqueous layer

The bulk of the aqueous component of the tears is not only composed of water, but contains numerous electrolytes, proteins, peptide growth factors, vitamins, anti-microbials, cytokines, immunoglobulins, and hormones. The composition of the tear film varies in response to environmental and bodily conditions. Electrolytes present in the tear film include sodium, potassium, magnesium, calcium, chloride, bicarbonate, and phosphate ions. These are largely responsible for modifying the osmolality of tears,[172] act as a buffer to maintain pH at a relatively constant level [173] and maintain epithelial integrity.[174]

To date, over 60 human tear proteins have been identified,[175, 176] of which the most predominant proteins are lysozyme, lactoferrin, and lipocalin. These proteins are secreted in response to an intracellular stimulus and the rate of secretion approximately matches the tear flow rate.[177, 178] Other important proteins of note include serum albumin, IgG, ceruloplasmin, transferrin, and monomeric IgA. Numerous peptide growth factors including EGF, HGF, TGF β are also found in the aqueous.[176] A recent study by Li et al[176] characterized the human tear proteome by using multiple proteomic analysis techniques, as described in Table 2-2.

Major components of human tear proteins
Identified proteins
(1) Albumin: Von Ebner's gland protein (Tear prealbumin), serum albumin
(2) Transferrin: serotransferrin precursor
(3) Lysozyme: lysozyme C precursor (EC 3.2.1.17) (1,4-beta-*N*-acetylmuramidase C), contribution of hydrophobic effect to the conformational stability of human lysozyme
(4) Ig: IgA, IgG, IgM, Ig alpha-1 chain C region, Ig alpha-2 chain C region, Ig lambda chain C region, Ig kappa chain C region, Ig heavy chain V-III region BRO/or TEI, immunoglobulin J chain, Ig heavy chain V-I region SIE, Ig mu chain C region, Ig heavy chain variable region, polymeric-immunoglobulin receptor precursor (Poly-Ig receptor) (PIGR)
(5) Cystatin: cystatin SN precursor (salivary cystatin SA-1), cystatin S precursor (salivary acidic protein-1) (Cystatin SA-III), cystatin SA precursor (cystatin S5), cystatin D precursor, cystatin C precursor (neuroendocrine basic polypeptide)
(6) Proline-rich protein: proline-rich protein 1, proline-rich protein 3 precursor, proline-rich protein 4, nasopharyngeal carcinoma-associated proline rich 4, proline-rich protein 5 precursor (proline-rich protein PBI)
(7) Lactoferrin
(8) Lipocalin
(9) Epidermal growth factor (EGF)
(10) Aquaporin 5
(11) α-Defensins
(12) Prolactin-inducible protein
(13) Mammaglobin B
(14) Phospholipase A, membrane-associated
(15) Extracellular glycoprotein lacritin precursor
(16) Lipophilin A precursor (secretoglobin family1D member 1)
(17) Beta-2-microglobulin precursor (HDCMA22P)
(18) Antileukoproteinase 1 precursor (ALP)
(19) Brain-specific angiogenesis inhibitor 3 precursor
(20) Aspartyl aminopeptidase (EC 3.4.11.21)
(21) G-rich sequence factor-1 (GRSF-1)
(22) 5'-AMP-activated protein kinase, catalytic alpha-2 chain (EC 2.7.1.-) (AMPK alpha-2 chain)
(23) Oxygen-regulated protein 1
(24) Clusterin precursor (Complement-associated protein SP-40,40)
(25) Mesothelin precursor (CAK1 antigen)
(26) Endothelial transcription factor GATA-2
(27) Nuclear RNA export factor 1 (Tip associating protein) (Tip-associated protein)
(28) Leucine-rich primary response protein 1 (follicle-stimulating hormone primary response protein)
(29) 60S ribosomal protein L18a
(30) Leucine-rich repeat transmembrane protein FLRT3 precursor
(31) Chloride intracellular channel protein 2 (XAP121)
(32) Basic salivary proline-rich protein 4 allele M
(33) Deleted in malignant brain tumors 1 isoform a precursor
(34) KFLA590
(35) Hypothetical protein
(36) Similar to common salivary protein 1
(37) Phospholipid transfer protein precursor
(38) Hypothetical protein

Table 2-2: Major components of human tear protein

Reprinted from Clinica Chimica Acta. Ohashi Y, Dogru M, Tsubota K. Laboratory findings in tear fluid analysis.[168] Clin Chim Acta 2006;369:17-28, with permission from Elsevier.

2.8.2.3 Functions of the aqueous layer

The functions of the aqueous layer include:

1. This is quantitatively the most important layer.
2. It is responsible for creating a conducive environment for the epithelial cells of the ocular surface, carrying essential nutrients and oxygen to the cornea, allowing cell movement over the ocular surface.[171]
3. Lysozyme, [171] lipocalin, lactoferrin and IgA are responsible for antimicrobial activity.[179, 180]
4. Washing away debris, toxic substance and foreign bodies.
5. Many of the growth factors that are present in the aqueous phase play a significant role in corneal physiology. [171]

2.8.2.4 Physical properties of the aqueous layer

The normal pH value of the tear film is between 7.14 and 7.82, with a mean value of 7.4-7.5, which is similar to plasma pH.[181]

2.8.2.5 Tear dynamics and drainage

The tears are directed from the upper temporal fornix to the lacrimal puncta in different ways. Tears move downward by gravity at the lateral canthus to form the lower marginal tear strip, capillary attraction then moves tears into the punctum and vertical section of the canaliculus, and finally the lid movement during the blink forces the tears in the puncta. The rapid closure of the temporal end of the palpebral aperture during the blink aids the nasal movement of tears. The same blinking movement forces the tears into the lacrimal sac through the canaliculi from each of the lacrimal puncta. The lacrimal sac collapses after the blink due to the relaxation of the orbicularis, thus forcing the tears into

the nasolacrimal duct. From the nasolacrimal duct, the tears are finally excreted into the nasal cavity. The basal tear production of the tear film is 1-2 µl/min and the turnover rate is approximately 16%/min in normal subjects.[55, 182]

2.8.2.6 Methods to assess major aqueous components (proteins)

Several assays are available to determine the level of major lacrimal gland proteins such as lysozyme[183, 184] and lipocalin in tears. Various biochemical assays including Enzyme-Linked Immunosorbent Assay (ELISA), High Performance Liquid Chromatography (HPLC) and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) are useful methods, due to their high sensitivity, accuracy and ability to target specific proteins. Other techniques, including capillary electrophoresis (CE), surface-enhanced laser adsorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), protein chip array methods, fluorescent antibody and ultrastructural techniques have all been used to study proteins.[168]

2.8.2.7 Aqueous layer in dry eye

Lysozyme levels decrease with age and in dry eye syndromes.[185] Sjogren's syndrome patients show a decrease in lysozyme, lactoferrin and EGF levels in tears.[183, 186] McCulley[187] reported that blepharitis patients with clinically diagnosed KCS had a lower mean tear film lysozyme concentration compared to either blepharitis patients with no KCS or control subjects. Deficiency of tear lipocalin can lead to the formation of mucous strands and cause tear film instability.[149] In a study of intolerant contact lens wearers,[167] it was found that tear lipocalin concentration was significantly elevated compared to a control group of tolerant wearers. The concentration of Aquaporin 5 (AQP5), which is a

selective water channel protein, was significantly increased in tears of Sjogren's syndrome patients, compared with normal controls.[188]

2.8.3 The mucous layer

2.8.3.1 Production of the mucin layer

The primary source of tear film mucins is from the goblet cells of the conjunctiva and the crypts of Henle in the conjunctival fornices. A secondary source of mucin is from the squamous epithelial cells of the ocular surface (cornea and conjunctiva), [135, 189-191] with a small contribution from the lacrimal gland. Goblet cell mucin forms a gel in the deepest layer of the tear film, while soluble mucin is found in the aqueous layer.[192] Ocular mucins influence the tear-film break up time and play a major role in stabilizing and spreading the tear film,[193-195] and also play a major lubrication function.[193-195]

2.8.3.2 Functions of the mucin layer

There are several key functions of the mucin layer, which include:

1. The most important function of mucin is lubrication, facilitating the eyelid margins and palpebral conjunctiva to slide smoothly over one another during blinking and ocular rotational movements.[196]
2. The corneal surface is wettable, however when non-wetting occurs in areas of the cornea, mucus plays an important role in overcoming this hydrophobicity.[196]
3. Mucus threads cover foreign bodies with a slippery coating, protecting the cornea and conjunctiva from abrasion.
4. Mucus also helps in wetting the ocular surface and in glycocalyx formation.[135]
5. The ocular surface glycocalyx acts as a barrier to pathogens.[197]

2.8.3.3 Composition and properties of mucins

Mucins are defined as high molecular weight glycoproteins, that have at least 50% of their mass is carbohydrate, O-linked to serine and threonine residues present within tandem repeats of amino acids in their protein backbone.[194, 198-202] They are hydrophilic in nature. The carbohydrate chains account for about 70% to 80% of the dry weight of mucins.[194, 198-202] The molecular mass of mucins range from 3×10^5 to over 4×10^7 kDa.[194, 198-202]

To date, at least 20 distinct human mucins have been cloned (MUC1– MUC20, including 3A, 3B, 5AC, and 5B).[203-212] Mucins are classified as either transmembrane or secretory mucins. Secreted mucins can be further sub-classified as gel-forming or soluble, based on their ability to form polymers. Of these, MUCs 1, 3A, 3B, 4, 12, 13, 15, 16, 17 and 20 have been characterized as membrane associated. MUC 2, 5AC, 5B, 6, 7 and MUC9 are classified as secretory mucins. In this category, MUC 2, 5AC, 5B, 6 are categorized as gel-forming mucins and MUC7 & MUC9 are classified as soluble mucins.

Demonstration of the expression of a specific mucin mRNA, as determined by northern blot or PCR and in situ hybridization is often considered as gold standard.[201] For the demonstration of mucin protein presence and distribution, it is necessary to use antibodies that have well-documented specificity.[201]

With the above criteria, MUC1, MUC4, and MUC16 have been identified as membrane bound mucins in the eye and MUC5AC, MUC2 and MUC7 (small amounts) have been identified as secretory mucins. Many of the membrane-associated mucins are shed from the epithelial surface and are present as soluble forms in extracellular fluids.

Several reports have shown alterations of mucin products in the ocular surface in dry eye.[213-216]

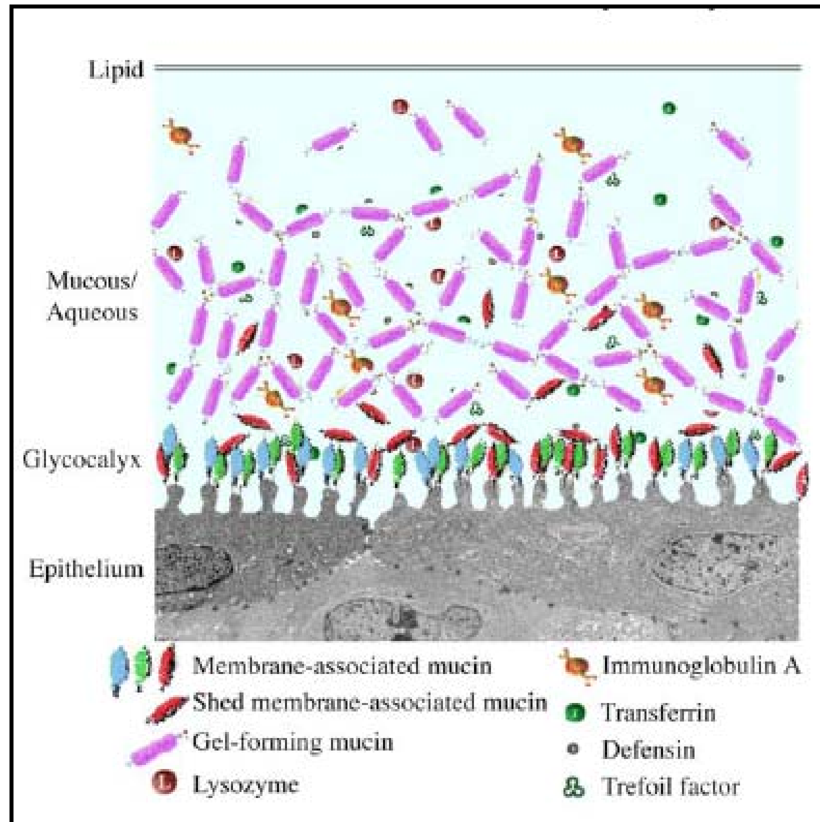

Figure 2-8: Diagram of the tear film and its interface with the ocular surface epithelium

Reprinted from Experimental Eye Research. Gipson IK. Distribution of mucins at the ocular surface.[201] Exp Eye Res 2004; 78(3)379-88, with permission from Elsevier.

2.8.3.3.1 Transmembrane mucins

Transmembrane mucins (Figure 2-9) contain hydrophobic, membrane-spanning domains in their carboxyl-terminal region, which anchor them to the apical surface of conjunctival and corneal epithelial cells, facilitating formation of the ocular surface glycocalyx.[217] Inatomi et al[191] demonstrated that MUC1 is expressed by both conjunctival and corneal epithelial cells, and that MUC4 is expressed only by the conjunctival

epithelium.[200] Later studies using sensitive assay techniques have detected MUC4 mRNA in corneal epithelia at reduced levels.[136] Recently, MUC16 mRNA and protein expression have been identified in conjunctival and corneal epithelia,[218] and MUC13, MUC15, and MUC17 mRNA have been detected in the epithelium of the conjunctiva.[219]

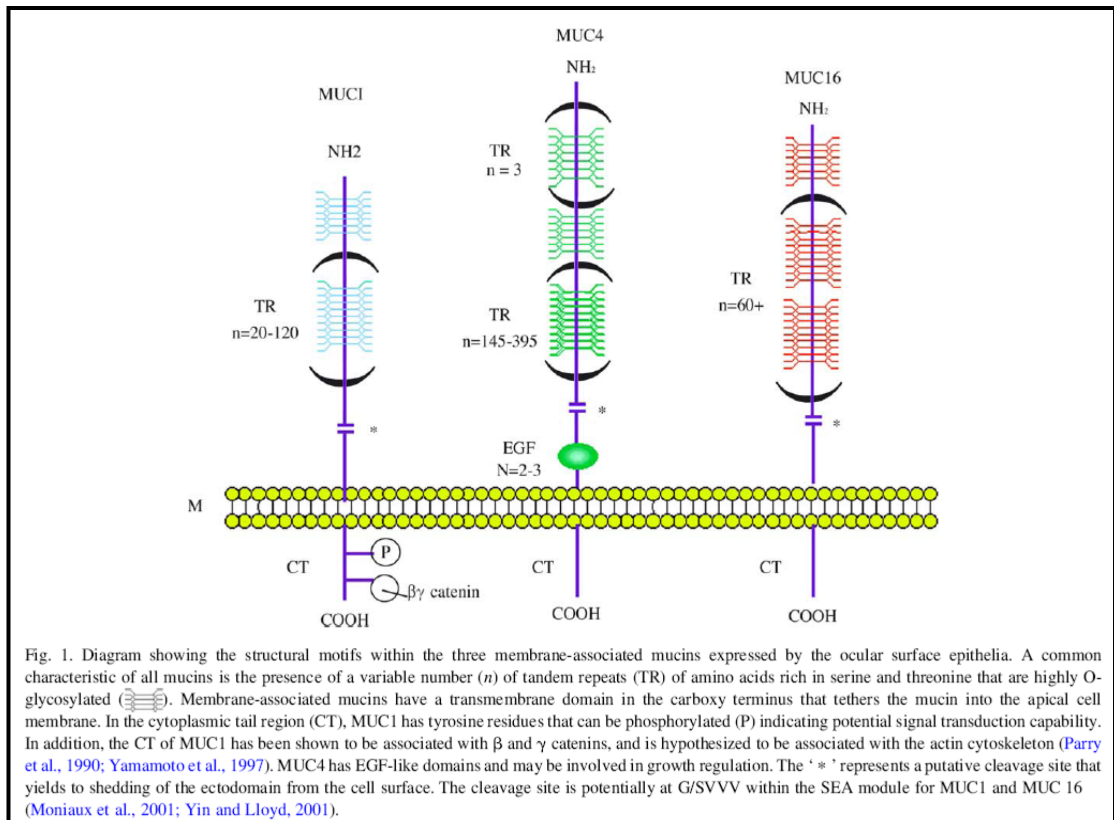

Fig. 1. Diagram showing the structural motifs within the three membrane-associated mucins expressed by the ocular surface epithelia. A common characteristic of all mucins is the presence of a variable number (*n*) of tandem repeats (TR) of amino acids rich in serine and threonine that are highly O-glycosylated ( ). Membrane-associated mucins have a transmembrane domain in the carboxy terminus that tethers the mucin into the apical cell membrane. In the cytoplasmic tail region (CT), MUC1 has tyrosine residues that can be phosphorylated (P) indicating potential signal transduction capability. In addition, the CT of MUC1 has been shown to be associated with β and γ catenins, and is hypothesized to be associated with the actin cytoskeleton (Parry et al., 1990; Yamamoto et al., 1997). MUC4 has EGF-like domains and may be involved in growth regulation. The ' * ' represents a putative cleavage site that yields to shedding of the ectodomain from the cell surface. The cleavage site is potentially at G/SVVV within the SEA module for MUC1 and MUC 16 (Moniaux et al., 2001; Yin and Lloyd, 2001).

Figure 2-9: The structure of membrane associated mucins

Reprinted from Experimental Eye Research. Gipson IK. Distribution of mucins at the ocular surface, Exp Eye Res 2004; 78(3)379-88, with permission from Elsevier.

2.8.3.3.2 Secretory mucins

The gel-forming mucins are the largest glycoproteins known, with genes of 15.7 to 17 kb and deduced proteins of approximately 600 kDa. The hydrophilic character of secreted mucins, which results from their heavy glycosylation, helps to hold fluids on epithelial surfaces. MUC5AC, the major gel-forming mucin in tears, is secreted by

conjunctival goblet cells.[200, 220] It has also been reported that MUC2, also a gel-forming mucin, is present in tears, but at levels several orders of magnitude lower than MUC5AC.[221] MUC7 is a small monomeric, soluble mucin produced by the lacrimal gland and conjunctiva.[222]

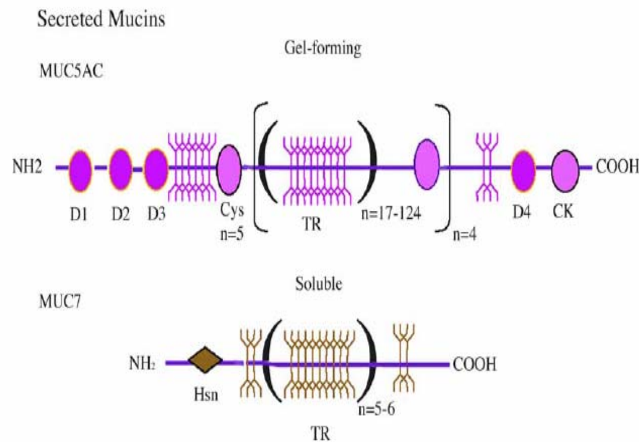

Fig. 2. Diagram of structural motifs of the two types of secreted mucins, the gel-forming MUC5AC and the small soluble, MUC7, secreted by conjunctival goblet cells and lacrimal gland, respectively. MUC5AC contains four cysteine-rich domains (D1, D2, D3 and D4), which are similar to the D domains of von Willebrand Factor. The D domains flank the tandem repeat region (TR) and provide sites for disulfide cross-linking to allow multimerization between MUC5AC molecules to form a mucin network. The function of additional cysteines (Cys) and a cysteine knot (CK) is not clear. The small soluble mucin, MUC7 is monomeric and has a histatin-like domain (Hsn) near its amino terminus, which is believed to have antifungal or anticandidacidal activity (Gururaja et al., 1999; Liu et al., 2000).

Figure 2-10: The structure of secreted mucins

Reprinted from Experimental Eye Research. Gipson IK. Distribution of mucins at the ocular surface, Exp Eye Res 2004; 78(3)379-88, with permission from Elsevier.

2.8.3.4 Methods to quantify ocular surface mucins

2.8.3.4.1 Impression cytology for mucin mRNA and protein analysis

Impression cytology allows for the collection of apical and subapical cells of the conjunctival epithelium, as well as goblet cells. It is a simple, non invasive technique to collect 2-5 layers of conjunctival epithelial cells and is explained in greater detail in

Chapters 3, 8 and 9. This method is particularly useful for the analysis of mucins, as membrane-associated and gel-forming mucins are present in the suprabasal cells of the conjunctival and in the goblet cells, respectively.[223]

Cloning and characterization of mucin genes has facilitated the use of polymerase chain reaction (PCR) to determine the mucin mRNA repertoire in cells collected by impression cytology. The only drawback of this method is that the yield of starting material may be low, which in many cases requires pooling of the left and right eye impression cytology samples. RNA isolation from the filter discs that are used to collect the sample, reverse-transcription into cDNA, and real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of the mucin mRNA content can be determined.[108] Impression cytology is also suitable for mucin protein analysis by immunofluorescence and flow cytometry. Brush cytology (for mRNA and protein analysis) is comparatively invasive.[224]

2.8.3.4.2 ELISA assay and immunoblot assay of tears

Polyacrylamide gel electrophoresis (PAGE) has been used for many years for analysis of proteins in human tears. However, the heavy glycosylation and sticky nature of mucins remains a challenge when attempting to study them. It is thus appropriate to partially deglycosylate the sample to facilitate access of the antibody to the mucin apoprotein. The amount of membrane-associated mucin shed into the tear film by the ocular surface epithelia can be assessed by immunoblot analysis. The presence of MUC4 and MUC1 in tears has also been detected by immunoblot analysis of human tear fluid collected by Schirmer strips.[225]

2.8.3.4.3 Conjunctival biopsy for immunolocalization and in situ hybridization

Conjunctival biopsies from living donors for mucin analysis, even though invasive, allow localization of the specific sites of mucin mRNA synthesis and protein expression through all the cell layers of the epithelium. Following biopsy, distribution of mucin mRNA and protein can be analyzed by in situ hybridization (ISH) and immunological techniques, respectively.[191, 200] Immunohistochemistry, when performed individually, may lead to errors due to poor characterization of the mucin antibodies and to the sticky nature of mucins, which induces nonspecific binding.

Researchers often prefer to use a combination of ISH and immunohistochemistry to demonstrate mucin distribution. The presence and distribution of MUC1 and MUC16 transcripts have been demonstrated by ISH in corneal and conjunctival epithelia.[191, 218] The presence of tandem repeated sequences in the nucleotide sequence of mucins has facilitated their analysis by ISH, since probes to the tandem repeat bind at multiple sites along the mucin mRNA detection.[226]

2.8.3.5 Mucins in dry eye syndrome

Studies indicate that mucin gene expression and translation, as well as mucin post-translational processing are affected in dry eye conditions.[201] Real-time quantitative PCR have shown a significant decrease in RNA transcripts for MUC5AC in the conjunctival epithelium of patients with Sjogrens syndrome, compared with normal individuals.[108] Flow cytometry analysis has also shown a decrease in the percentage of MUC5AC-positive conjunctival cells.[215] Protein levels of MUC5AC assessed by ELISA are also significantly reduced in the tear fluid in dry eye.[108] Alterations in membrane-associated

mucins are noticed in non-Sjogren's syndrome dry eye[227] and Sjogren's syndrome dry eye.[214]

From samples obtained via conjunctival impression cytology, a significant difference in the binding pattern of an antibody against a carbonate epitope antibody (H185) carried by MUC16 to conjunctival epithelium in normal eyes when compared with those of patients with non-Sjogren's dry eye has been recently demonstrated.[213] A trend towards decrease in MUC1 mRNA was also noted. It was also demonstrated that during keratinization of the ocular surface epithelia, the pattern of expression of glycosyltransferases that initiate O-glycosylation on mucins is altered, which may lead to alterations in carbohydrate structures on the mucins.[228] Table 2-3 summarizes a body of information on human tear film in various dry eye conditions

Table 2-3: Summary of information on human tear film in various dry eye conditions (adapted from DEWS report)

	KCS	NSS	SS	MGD	Androgen deficiency	CL dry eye	References
Tears - clinical assessment							
↓ Volume ↑ Osmolality	X	X	X	X	X	X	75, 105, 110, 229, 230
↑Evaporation	X			X			1, 231-233
↓ Meniscus	X	X	X	X	X	X	75, 234, 235
↓ BUT ↑ Surface tension	X	X	X	X	X	X	75, 152, 236-239
Tears – analytical assessment							
Mucins							
↓ Glycoproteins, MUC5AC	X		X	X			107, 108
Lipids							
Changes in lipid patterns, distribution			X	X			240, 241
↓Polar lipids, lipid layer, ↑ evaporation	X						151, 242
Inflammatory mediators							
Pro inf. cytokines (IL 1, 6, 8, TNF α)			X	X			186, 243, 244
Proteins							
Change in proteins	X						188, 245
↑ Plasmin levels	X						246
↑ MMP's				X			247, 248
↑ Inflammatory markers	X			X			249
↓ Lysozyme and lactoferrin							250
↑ Phospholipase A2	X					X	167

KCS- keratoconjunctivitis sicca, NSS – non-Sjogren's syndrome, SS - Sjogren's syndrome, MGD – meibomian gland dysfunction, CL dry eye – contact lens related dry eye.

2.9 The ocular surface

2.9.1 Conjunctiva

The conjunctiva is a mucous membrane that covers the inner surface of the upper and lower lids and extends to the limbus, on the surface of the globe. The conjunctival mucous epithelium, a stratified squamous non-keratinizing epithelium of 2 to 10 cell layers, is critical in protecting the eye from external stimuli and maintaining a healthy ocular surface.[251] The limbal epithelium is believed to be about 10 cells deep. The conjunctival epithelium has secretory goblet cells.[251] In conjunction with mucin secreted by conjunctival goblet cells (MUC5AC), the membrane-associated mucins of apical corneal and conjunctival epithelium (MUC1, MUC4 &MUC16) protect and hydrate the ocular surface.[199, 218, 220] The inflammatory cytokines secreted by the conjunctival epithelium are involved in the pathogenesis of ocular surface diseases such as keratoconjunctivitis sicca.[186]

2.9.2 Corneal epithelium

The corneal epithelium comprises an outer stratified squamous non-keratinized epithelium and inner stroma, with keratocytes and cuboidal endothelium. There are 5 to 7 corneal epithelial cell layers. The outermost apical cell layer has microplicae and exhibits a prominent filamentous glycocalyx, which plays a vital role in mucin and tear film spreading over the surface of the eye.[251] The membrane-associated mucins are the major components of the glycocalyx, along the apical cell-tear film interface.[194, 199] Table 2-4 summarizes the information on human ocular surface in various dry eye conditions.

Table 2-4: Summary of information on human ocular surface in various dry eye conditions (adapted from DEWS report)

	KCS	NSS/Aqueous	SS	CL wear	LASIK	References
Mucins						
↓ Goblet cells	X	X	X	↑	X	252-257
↓ MUC5AC	X		X			107, 108
Alteration in mucin glycosylation	X			X		216, 258-260
Change in membrane associated mucins		X	X			108, 213, 253, 258
Conjunctival epithelial changes						
↑ Stratification	X					228, 261
Epithelial proliferation			X			262
Changes to nuclear structure	X		X			263-265
↑Apoptosis	X	X	X			266
Changes in innervation		X	X		X	267-270
↑ Infections	X					250, 271
↑ Keratinization			X			272-274
Inflammatory markers on epithelial cells	X	X	X			275, 276
Ocular surface damage – NaFl, LG, RB staining	X	X	X	X	X	1, 277-279

KCS- keratoconjunctivitis sicca, NSS – non-Sjogren's syndrome, SS - Sjogren's syndrome, MGD – meibomian gland dysfunction, CL dry eye – contact lens related dry eye.

2.10 Methods to study dry eye

Complete clinical and analytical assessment of the tears and ocular surface is necessary to diagnose the severity and monitor the development and progression of dry eye. A variety of tests and diagnostic criteria are used by clinicians and researchers to characterise dry eye. Large epidemiological studies conducted in the past 10 years have

chosen different diagnostic criteria to study dry eye, which complicates comparisons and development of cut off values in its diagnosis. Recent reports have suggested strict templates and criteriae to help clinicians and researchers accurately confirm a dry eye diagnosis.[280]

Symptoms play a key role in the diagnosis, and hence validated dry eye questionnaires are valuable tools for routine screening procedure. Tear evaluation procedures include tear osmolality tests, TBUT and tear function tests. Evaluation of tears and ocular surface tissue samples to study inflammatory components in tears in dry eye still remains a challenge, due to the inadequate volume of tears or starting material (epithelial cells).

The global features of dry eye can be studied by utilizing the following types of diagnostic tests:

2.10.1 Dry eye questionnaires (DEQ)

It is difficult to arrive at a specific diagnostic standard for dry eye, in both clinical and research settings. Clinicians typically rely on case history and subjective symptoms to diagnose and categorize dry eye.[281, 282] There are a variety of questionnaires available,[88, 283-288] ranging from single item score questionnaires[289] to extensive questionnaires (eg. 57 questions)[124] targeting a variety of areas, including diurnal variation[237, 286, 290] of commonly occurring symptoms. The epidemiology subcommittee of the DEWS workshop has identified questionnaires that are validated and used in large studies.[65] Dry eye symptom questionnaires are also useful in assessing responses to dry eye therapy and DEQ's are discussed in more detail in chapter 4.

2.10.2 Assessment of tear film stability

Tear film stability has traditionally been assessed using the tear break-up time (TBUT) following the instillation of sodium fluorescein. Although this method is easy to perform, there are some disadvantages including variations in the concentration, pH of fluorescein, volume of fluorescein used, presence of preservatives and the invasiveness of the procedure itself.[291] Instillation of fluorescein in itself alters the quality and quantity of the tear film.[292] A non-invasive approach to measuring TBUT in the diagnosis of dry eye has been reported to be valuable for assessment of TBUT and has shown a high sensitivity and specificity.[293, 294] This method allows the evaluation of the tear film by eliminating the physical disturbance of the tear film from the instillation of fluorescein, along with the possibility of reflex tearing.[291] The exact mechanism behind the tear breakup is not clearly understood and three different hypotheses of tear breakup have been proposed, as described in section 2.4.1

2.10.3 Assessment of tear osmolality

Tear osmolality is often considered a "gold standard" in the evaluation of subjects with dry eye.[295-297] As an objective method, hyperosmolality is a single attractive parameter for characterizing dry eye. However, a lack of available equipment and the fact that most osmometers require a large volume of tears (typically 5-10µl),[298, 299] limits its use in many dry eye subjects, particularly those with severe disease.[299, 300] A recent study based on a meta analysis on published data in determining referent values for diagnosis of dry eye, has shown the cut off value to be 315.6 mOsm/Kg.[301] Tear osmolality is discussed in more detail in chapter 6.

2.10.4 Assessment of ocular surface staining

The use of vital dyes, including fluorescein and lissamine green, to grade ocular surface staining using different quantification methods is commonly used in dry eye studies.[1, 277 278, 279] Among the various quantitation scales available, three are widely accepted and used in dry eye studies, namely the van Bijsterveld scale,[278] the Oxford system[279] and the CLEK system.[1, 277]

2.10.5 Analytical tests of tears and ocular surface

Samples of tears collected with fine glass capillary tubes demonstrate higher lacrimal gland proteins when compared to invasive collection methods such as those using filter paper and cellulose sponges. These latter methods stimulate the conjunctiva, induce serum leakage, and result in a higher proportion of plasma proteins.[168] The relative proportions of the proteins present in an individual tear sample depend on the method of tear collection.[302] Non invasive techniques (including impression cytology and minimally invasive brush cytology) are useful to study inflammatory biomarkers. Impression cytology is discussed further in Chapters 3, 8 and 9.

2.11 Treatment and management of dry eye

Despite the high prevalence of dry eye, it remains a condition without complete cure.[51] The conventional current management strategy for dry eye management is to prescribe lubricant eye drops to provide temporary symptomatic relief,[63, 303, 304] in conjunction with surgical procedures and, more recently, pharmacological therapy to treat any underlying inflammation.

2.11.1 Tear supplements and lubricants

Tear supplements or ocular lubricants are typically hypotonic or isotonic buffered solutions, which contain electrolytes, surfactants and various viscosity-enhancing agents. Tear supplements should ideally be preservative free to avoid the side effects of commonly used preservatives such as benzalkonium chloride (BAK)[305-308] and EDTA.[309, 310] Solutions containing electrolytes and ions such as potassium[311] and bicarbonates[312] have also proven to aid in treating dry eye. Viscosity-enhancing agents[313] (macro molecular complexes such as hydroxyl methylcellulose - HMC) and hypo-osmotic artificial tears[314] are also used to alleviate symptoms of ocular dryness.

2.11.2 Tear retention

Punctal plugs[315-319] (absorbable and non-absorbable) are indicated for symptomatic patients with very low Schirmer scores.[316] Contact lenses have proven to be useful in certain severe dry eye conditions by improving comfort, vision and healing of corneal epithelial defects and hence decreasing corneal epitheliopathy.[320-323] The use of moisture chamber goggles have demonstrated an increase in the periocular humidity[324, 325] and hence tear film thickness.[326]

2.11.3 Anti inflammatory therapy and biological tear substitutes

Based on the current understanding of dry eye, inflammation is one of the key components in the pathogenesis of dry eye. Anti-inflammatory agents such as cyclosporine, corticosteroids, and tetracyclines are used in the treatment of certain cases of dry eye. Clinical trials conducted by Brignole et al[327] and Turner et al[328] using cyclosporine drops have shown a decrease in the expression of immune markers, apoptosis markers and cytokines (IL6), in the conjunctival epithelial cells of dry eyed

individuals. Another study has shown a T lymphocyte decrease in conjunctival tissue following the use of cyclosporine.[329] Corticosteroids have proven useful in the management of patients with Sjogren's syndrome[330, 331] and KCS conditions.[332, 333] The antibacterial and anti-inflammatory properties of tetracycline makes it clinically viable in patients with acne rosacea,[334] meibomian gland dysfunction[335] and chronic blepharitis.[336, 337]

The use of biological fluids such as serum[338-341] (used mainly in severe dry eye disease) and saliva (salivary mandibular gland transplantation[342, 343]) has been reported in novel studies looking at dry eye treatment.

2.11.4 Secretogogues

There is a list of agents/secretogogues under investigation that may stimulate tear secretion and prove to be useful in dry eye treatment. Certain agents stimulate mucin secretion (gefarnate,[344-346] rebamipide[347] and ecabet sodium[348]), in particular MUC1 (15(S)HETE).[225, 349-352] Diquafosol is a potent aqueous and mucin secreting agent in animals[139,353-355] and humans[356, 357] and reduces ocular surface staining.[358]

2.11.5 Essential fatty acids

Omega 3 fatty acids are known to inhibit the synthesis of lipid mediators and prevent the production of cytokines (IL1α and TNFα).[359, 360] Orally administered essential fatty acids have demonstrated improvements in ocular irritation symptoms and signs.[361]

2.11.6 Environmental strategies

Environmental effects have been explained in section 2.2.2.1. Avoidance of systemic medications such as antidepressants and antihistamines may prevent symptoms

of ocular dryness. Avoiding low humidity or air-conditioned areas causing environmental stress hence prevent increased evaporation of tears or less production.[35, 362, 363] Good blinking habits[364] and the use of VDT terminals below eye level decreas the interpalpebral aperture exposure to environment and hence prevent evaporation of tears.[119, 364-366]

In this chapter, a broad over view of dry eye was discussed. The next chapter will focus on the methods undertaken in this PhD project and the subsequent chapters will focus on the clinical and analytical studies performed in a unique participant pool.

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3 Materials and Methods

In this chapter, the number of participants, inclusion and exclusion criteria, the procedures conducted during the study visits and the instruments used will be described.

3.1 Subjects

Informed consent was obtained from all participants prior to enrolment in this investigator masked study. This work received approval from the Office of Research Ethics at the University of Waterloo, Waterloo, Ontario, Canada (ORE #11003). All subjects were treated in accordance with the tenets of the Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects adopted by the 18th World Medical Association General Assembly Helsinki, Finland, June 1964).[1]

A total of 86 participants were enrolled, of which 82 successfully completed the study. Four participants did not complete the study due to the reasons presented in Table 3-1. Tear samples were collected wherever possible.

Table 3-1: List of participants who discontinued from the study

Participant ID	Reason for discontinuation
15, 58, 59	Uncomfortable with impression cytology technique
25	Allergy to topical anesthetic

3.1.1 Inclusion criteria

Participants were eligible for entry into the study as a dry eyed participant if she:

1. Was a post–menopausal woman (menses ceased more than 12 months prior to the start of the study).
2. Was at least 50 years of age and had full legal capacity to volunteer.
3. Had purchased over-the-counter eye drops for dry eye symptoms within the previous six months.
4. Had an oculo-visual examination in the last 2 years.
5. Had read and signed the Statement of Informed Consent when complete eligibility had been confirmed.

3.1.2 Exclusion criteria

Participants were ineligible for entry into the study if she:

1. Ceased menses due to autoimmune disorders, chemotherapy, pelvic irradiation or smoking.
2. Had confirmed Sjogren's syndrome.
3. Wore soft or rigid gas permeable contact lenses.
4. Had any clinically significant lid or conjunctival abnormalities, neovascularization, or corneal opacities.
5. Was aphakic.
6. Had corneal refractive surgery.
7. Had ocular surgery in the past year.
8. Was diabetic.
9. Had a clinical diagnosis of blepharitis.

10. Was taking any anti-glaucoma medication.
11. Was participating in any other type of clinical or research study.

The control group of participants were postmenopausal women (not on HRT) with no symptoms of dry eye (did not use any artificial tears) or any other anterior segment abnormality. In total, clinical procedures were performed on all 82 participants. These participants were sub-divided into various groups for performing the analytical procedures (tear analysis and conjunctival epithelial cell analysis for biomarkers). The details of each group of subjects for each study are listed in the relevant chapters.

The following sections in this chapter detail the various procedures, in the same order they were performed during the clinical visits.

3.2 Informed consent and completion of dry eye questionnaire

Informed consent was obtained from all the participants prior to enrolment in the study. Participants then completed three different dry eye questionnaires (DEQ):

a. Ocular surface disease index (OSDI)[2, 3] – appendix A
b. Single item score dry eye questionnaire (SIDEQ)[4] – appendix B
c. Indiana DEQ[5-7] – appendix C

OSDI scoring is based on a 0-100 scale, with the highest score representing greater disability.[2, 3] An OSDI score of 0-12 represents non dry eye; an OSDI score of 13-22 is categorized as mild dry eye and an OSDI score of 23-32 represents moderate dry eye.[2,3] The SIDEQ self assessment questionnaire[4] assessed the subjects' ocular discomfort due to symptoms of dryness on a 0-4 scale, ranging from "none" to "severe." Participants who reported "none" or "trace" symptoms were grouped in the non-dry eyed

group and the remainder were grouped into the dry eyed group. The DEQ includes categorical scales to measure the frequency, diurnal intensity, and intrusiveness of common ocular surface symptoms and usage of medication.[5-7] These will be explained in the relevant chapters.

Averaging and pooling of clinical and biological data is explained in detail in section 3.14.

3.3 Objective bulbar conjunctival redness

Objective bulbar redness was quantified using a SpectraScan PR650© Spectrophotometer (Photo Research Inc, Chatsworth, CA) (Figure 3-1). It is a table-top device that determines measurements of luminance and chromaticity by measuring the absolute intensity at each wavelength and then calculating the equivalent CIE u' (Commission Internationale d'Eclairage) value.[8, 9] Previous experiments have successfully used u' values to measure ocular redness.[8, 9] Higher u' values denotes greater bulbar conjunctival redness. The chin rest and head rest were cleaned using alcohol swabs (Isopropyl alcohol 70%, Becton and Dickinson Canada Inc. Oakville, Ontario). The subject sat at the photometer and looked at a fixation light to their left or to the right, such that the temporal conjunctiva was aligned with the instrument. The examiner looked through the eye piece and positioned the black measuring spot (approximately 19.63 mm^2) of the photometer approximately 2 mm from the temporal limbus on the temporal bulbar conjunctiva (Figure 3-2). The spectrophotometer was turned on just before the measurement and turned off immediately after, to ensure that the ocular surface temperature did not increase. Redness was measured three times on both eyes. Mean value (left and right eye) was recorded for use in subsequent analysis.

Figure 3-1: SpectraScan PR650© Spectrophometer

Figure 3-2: The black measuring spot of the photometer

The black measuring spot (approximately 19.63 mm2) of the photometer was aimed approximately 2 mm from the temporal limbus on the temporal bulbar conjunctiva to determine hyperemia

3.4 Non invasive tear break up time

Tear stability was assessed by performing a non invasive tear breakup time (NITBUT) evaluation using the ALCON Eyemap® model EH-290 topography system (ALCON, Inc., Forth Worth, Texas, USA). The instrument has a keratoscope unit that produces concentric rings of light, which are reflected off the cornea and imaged in a CCD camera (Figure 3-3). The chin rest and head rest were cleaned using alcohol swabs. Participants were comfortably seated with their head supported by the forehead and chin rest and looked at a fixation light at the centre of the concentric rings of light. Participants were asked to blink 3 times before each measurement was taken. NITBUT was determined by measuring the time taken for distortions or discontinuities to appear in the reflected image of the concentric ring pattern (Figure 3-4). The time (in seconds) for the tear-film to rupture (and thus distort the rings) was measured using a stopwatch, to the nearest 0.1 of a second. Three measurements were taken in each eye and averaged. The mean values obtained in both eyes were then averaged and was used for analysis purposes.

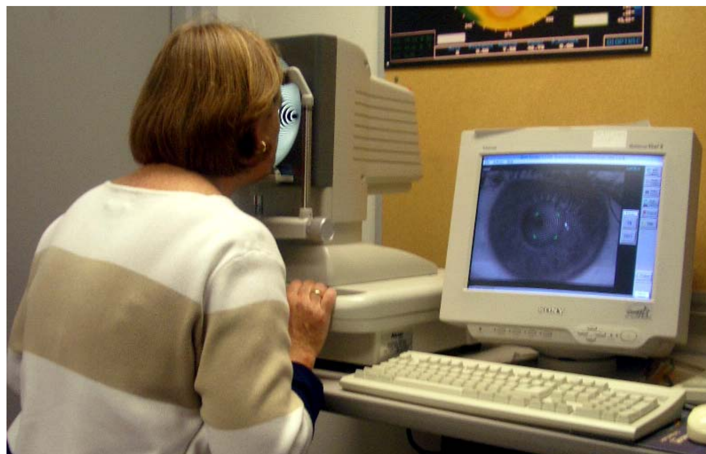

Figure 3-3 : ALCON Eyemap® model EH-290 topography system

Figure 3-4: Distortions or discontinuities in the reflected image of the concentric ring pattern

3.5 Phenol red thread test

Tear volume was assessed using the Phenol Red Thread (PRT) test (ZONE-QUICK, Showa Yakuhin Kako Co., Ltd. Tokyo, Japan). Two sterilized threads were contained in each aluminum package, as shown in Figure 3-5. Each thread was taken out by gently peeling the plastic film covering from the unsealed end of the aluminum package. The folded 3mm end of the thread was bent open at an angle that allowed easy placement onto the palpebral conjunctiva with forceps.

Figure 3-5: Phenol red thread test

The PRT was placed at a point approximately 1/3 of the distance from the lateral canthus of the lower eyelid, with the eye in primary position. The lower lid was pulled down gently, and the folded 3mm portion of the thread was placed on the palpebral conjunctiva at the position specified in Figure 3-5. Each eye was tested with the eyes open for 15 seconds. During the test the patients were instructed to look straight ahead and blink normally. After 15 seconds, the lower lid was gently pulled down, and the thread was gently removed with an upward motion. Care was taken to pull the eyelid down before removal of the thread to avoid discomfort. The length of the color change on the thread, which indicates the wetting length, was measured in millimeters from the very tip, regardless of the fold. No topical anesthetic was used. A stop watch was used to measure the time. Mean value (left and right eye) was recorded for use in subsequent analysis. A break period of 20 minutes was given before the next test was performed.

3.6 Tear collection using capillary tube

Participants were asked to sit on a reclining chair that was at the maximum reclining position. Participants were asked to incline their head towards the tear collector and then asked to look up and away from the tear collector. Single-use, graduated, disposable, sterile, smoothly polished, fine glass capillary tubes (Wiretol-Micropipettes, Drummond Scientific Co., Broomall, PA, USA) were used to collect tear samples (Figure 3-6). Approximately 6μL of tears were collected from the inferior tear meniscus of each subject. Tear collection was performed without corneal anaesthesia. Collections were performed as carefully as possible to reduce reflex tearing and, taking care to ensure that the lid margin and corneal surface were not touched (Figure 3-7). The time taken for drawing 6μL of the tear sample from each eye was noted. This sample was then carefully

transferred to a micro PCR tube (Axygen Maxymim™ Recovery microtubes, Axygen Scientific, Inc., California, USA) for use in osmometry, ferning test and protein analysis (explained below). The micro PCR tubes were placed on ice. Tears were then pooled together, vortexed very briefly, aliquoted into various volumes and immediately transferred to storage at -80°C.

Figure 3-6: Single-use, graduated, disposable glass capillary tubes (Wiretol-Micropipettes)

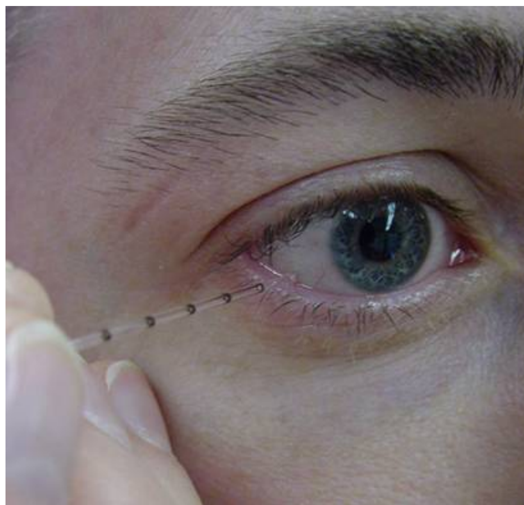

Figure 3-7: Tear collection using a disposable capillary tube

3.7 Tear osmolality measurements

The Model 3100 Tear Osmometer™ (Advanced Instruments, Inc. Norwood, MA, USA) is a diagnostic tool that quantifies tear osmolaltiy for ophthalmic applications. This instrument measures the osmolality of nanoliter-sized (0.5 μL) samples by freezing point depression. Approximately 0.5μL of the tears collected (as described in section 3.6) from the inferior meniscus of the right eye from each participant was transferred to a single-use, disposable polycarbonate capillary tube. This polycarbonate tube was placed on a syringe and the tear sample was dispensed onto a white tip (Figure 3-8). This white tip was then loaded on to the freezing point depression osmometer as shown in Figure 3-9. Tear samples varied in their time to freeze and the crystal patterns following freezing varied widely between participants. Based on the melting point of a 0.5 μL sample, the computer-based system automatically determines the osmolality. The final osmolality reading was displayed on the computer screen. The procedure was repeated twice and the mean value was taken for subsequent analysis. The instrument's operating range as stated by the manufacturer is 280-350 mOsm and its repeatability is ± 4mOsm.

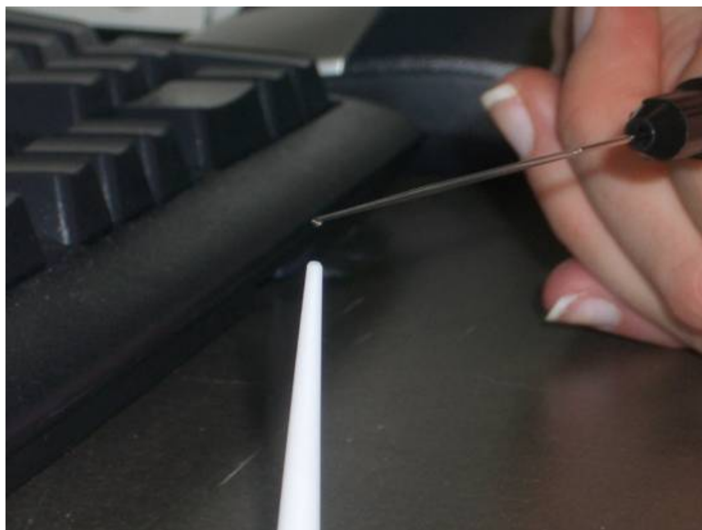

Figure 3-8: Loading tear samples for osmolality measurement

Figure 3-9: Model 3100 Tear Osmometer

3.8 Tear ferning

Approximately 0.5μL to 1 μL of tears collected (as described in section 3.6) from the inferior meniscus of the right eye from each participant was carefully pipetted from the tube. The Tear Ferning Test (TFT) was performed by dropping 0.5μL of the tear sample onto a clean glass microscope slide. This glass slide was allowed to dry at room temperature and evaluated at 10X magnification on a light microscope (Zeiss Axiovert 40 CFL, Carl Zeiss, Göttingen, Germany). The ferning reaction is caused by an interaction of electrolytes with macromolecules such as proteins or mucous polysaccharides.[10] The quality of ferning observed was based on the Rolando grading system,[11] which grades the ferning patterns from grades 1 (abundant ferning; Figure 3-10) through to grade 4 (no ferns) (Figure 6-4 in chapter 6). Photographs were taken immediately after drying to avoid misinterpretation in grading due to alterations in the ferning patterns due to effects of temperature and humidity.

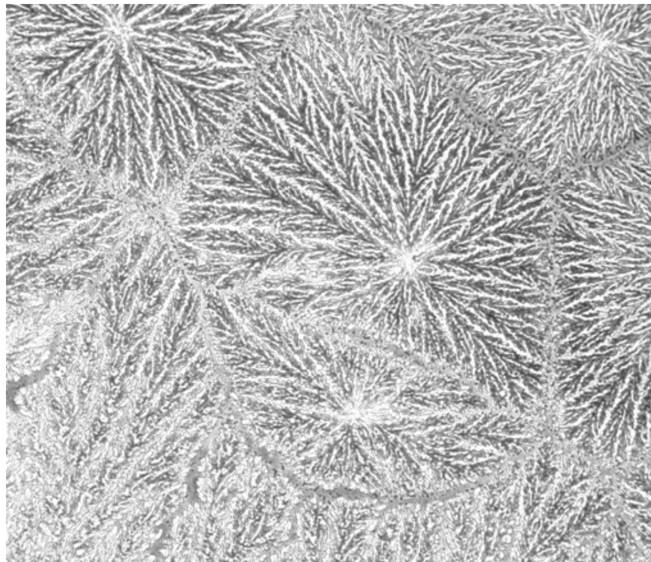

Figure 3-10: Uniform, abundant tear ferning, with no spaces between ferning (grade1)

3.9 Slit lamp evaluation

The subject was comfortably seated on a slit lamp biomicroscope (Zeiss slit lamp, Carl Zeiss, Göttingen, Germany) with their head supported by a forehead and chin rest. The chin rest and head rest were cleaned using alcohol swabs. Lid margins were assessed for meibomian gland dysfunction and vascularization. The level of temporal bulbar conjunctival hyperemia was graded by the examiner using a modified CCLRU scale, which uses a 0-100 scale (0 – negligible, 25 - trace 50 - mild 75 – moderate, 100 – severe).[8, 12, 13] Mean value (left and right eye) was recorded for use in subsequent analysis. All the parameters were assessed under diffuse white light with 16X magnification. Corneal and conjunctival staining was not performed during the slit lamp examination to avoid interference with the impression cytology procedure.

3.10 Collection of tears using eye wash technique

Tears were collected using the eye wash technique on a subset of participants (ID # 36 to 86 only). This technique allows the collection of tears from dry ocular surfaces which is commonly seen in moderate to severe dry eye patients. Participants were asked to sit on a reclining chair at the maximum reclining position. With head straight ahead and slightly tilted upwards the participants were asked to look down. The upper lid was held by one researcher and the lower lid held by another researcher. Sixty micro litres of sterile, physiological saline (0.9% NaCl) (Minims, Chauvin Pharmaceuticals Ltd, Romford, Essex, UK) in a sterile micropipette (modified in house to a finer, blunt tip) (VWR Cat# 14670-366), was instilled onto the superior bulbar region of the un-anaesthetized ocular surface. Participants were asked to move their eye to the left, to the right, up and down, without blinking (lids were held open), then were asked to repeat the

eye movements in order to mix the tear fluid content. Tear washes were collected from the inferior fornix of each eye using the sterile micropipette (Figure 3-11). The participants were asked to tilt their head towards the researcher while removing the eye wash solution, in order for the solution to pool in the lateral canthal region. The procedure was repeated with the other eye with a fresh sterile micropipette. Eye washes were pooled together, vortexed very briefly, then aliquoted into two samples of 5µL and the remainder volume was recorded. All the samples were stored immediately on dry ice. Eye wash samples were then transferred to -80°C freezer until further use.

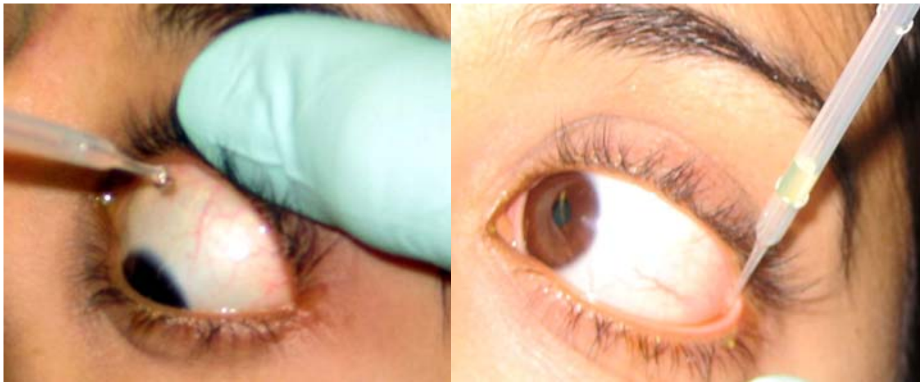

Figure 3-11: Collection of eyewash tears

3.11 Conjunctival impression cytology (CIC)

3.11.1 Method

Instruments {hole punch, flat forceps (Fisher, catalogue # XX62 000 06) and pointed forceps (VWR, catalogue # 25607-856)}, and surgical scissors were sterilized at 230°C for 9 hours. Gloves (High Five Products Inc, Chicago, Illinois) were worn by the researcher. Large round filter paper Millipore MF™ membrane filters 0.45µm (Millipore, catalogue # HAWP09000) were placed on Kimwipes in a laminar flow hood for sterility purposes. Direct pressure was applied to the large round filter paper membrane using the

10 mm diameter canvas punch until paper yields were obtained (Figure 3-12). The filter paper discs were then sterilized under UV for 5 minutes. Care was taken to adhere to the time period, to avoid the membrane becoming brittle. Using forceps, four discs were placed into 1.7 mL sterile eppendorf tubes (VWR, catalogue # 22234-048). These were used for performing conjunctival impression cytology. In addition to the 10mm diameter punch, 8mm and 9mm diameter punches were also used to obtain slightly smaller sized membranes for smaller horizontal visible iris diameters.

Figure 3-12: Large Millipore MF™ membrane filters were used to make discs of 10mm diameter for impression cytology

3.11.2 CIC procedure

Participants were comfortably seated in a bench-height laboratory chair with a back rest. With the participant's head slightly reclined upward, 1 drop of topical anaesthetic (Alcaine™ 0.5% proparacaine hydrochloride; Alcon Canada Inc, Mississauga, Canada) was instilled onto the superior bulbar region of each eye. Participants were asked to close their eyes. Following a wait period of 30 seconds, a second drop of topical anaesthetic was instilled onto each eye.

3.11.2.1Impression cytology of the superior bulbar region

Participants were asked to gaze down as far as possible. One researcher held the upper lid, while the other researcher placed an appropriately-sized pre-cut Millipore MF™ membrane filter gently onto the superior bulbar conjunctiva using the pointed forceps.

The membrane was tapped down gently with the same forceps and left on the eye for 5-8 seconds (Figure 3-13). The membrane was then gently removed from the eye with the flat edge forceps. The membrane from the right superior bulbar conjunctiva was immersed directly into an RNase free 2 ml tube containing 1 ml of RLT lysis buffer (Qiagen RNeasy mini kit Cat# 74106) that had 1% β-mercaptoethanol added immediately prior to use.

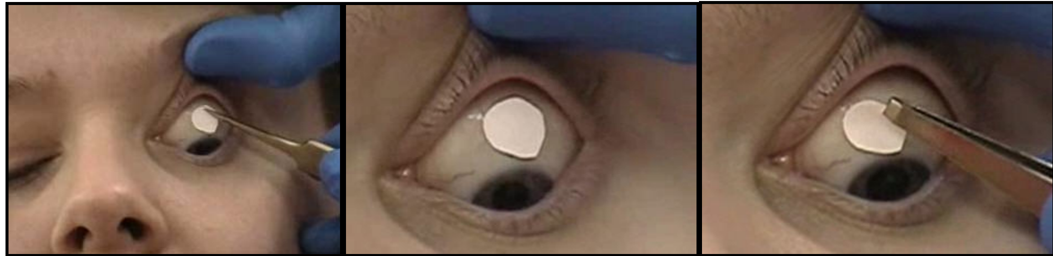

Figure 3-13: Impression cytology of the superior bulbar conjunctiva

3.11.2.2Impression cytology of the right temporal bulbar region

Participants were asked to look down as far as possible. One researcher held the upper lid open and then the participant was asked to stare directly ahead and to the extreme left gaze while the second researcher held the lower lid and then, using the pointed forceps, placed the membrane onto the temporal bulbar conjunctiva (Figure 3-

14). The membrane was tapped down gently with the pointed forceps and the membrane was left on the eye for 5-8 seconds.

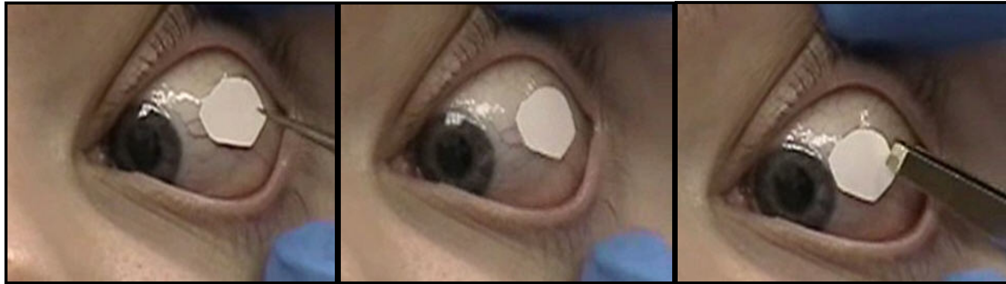

Figure 3-14: Impression cytology of the temporal bulbar conjunctiva

The membrane was gently removed from the eye with the flat edge forceps. This was immersed into the same tube used for the right superior bulbar conjunctiva membrane. Care was taken to ensure both membranes were soaked in the lysis buffer and kept on dry ice.

The same procedure was repeated for impression cytology of the left superior bulbar conjunctiva. The membrane collected from the left superior bulbar conjunctiva was placed into an empty RNase free 2ml tube. Similar steps described above for the collection of samples from right temporal bulbar conjunctiva was repeated for the left eye also. The participants were asked to look up and to the extreme right to collect the temporal bulbar conjunctival samples from the left eye. This membrane was placed into the same tube that contained the left superior bulbar conjunctiva membrane (Figure 3-15). The CIC membranes from the left eye were used for protein analysis. This tube was placed on dry ice immediately. Both tubes were transferred to -80ºC freezer until processing.

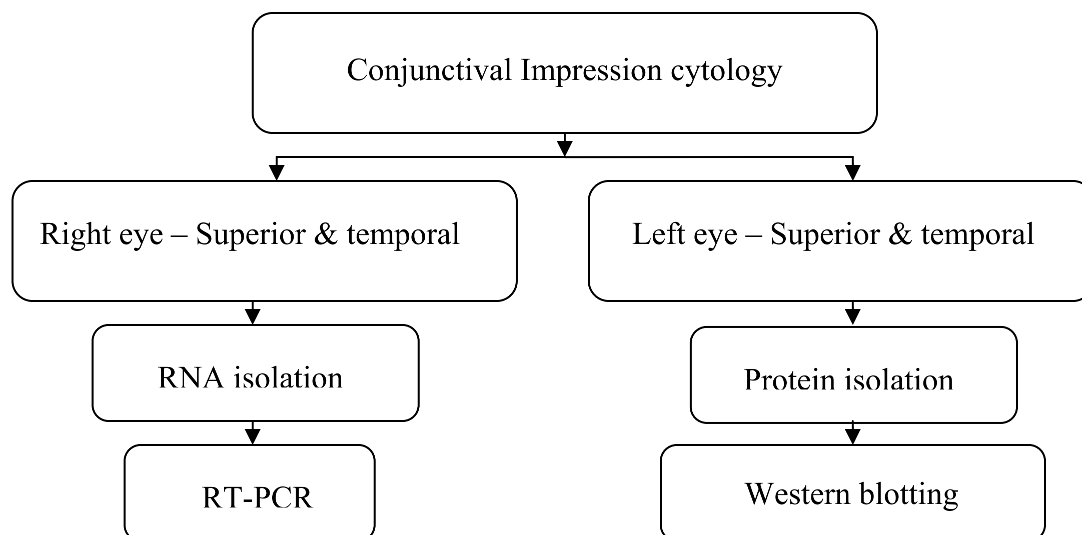

Figure 3-15: Flowchart showing the schematic of sample processing from the CIC disc from left and right eye

Following impression cytology, two drops of TheraTears® (Advanced Vision Research, Woburn, MA) lubricant tear drops were placed into both left and the right eyes. Artificial tears were administered immediately to alleviate the symptoms of mild discomfort post-procedure.

3.12 Corneal and conjunctival staining

Before all the participants exited the study, the eyes were stained with sodium fluorescein (NaFl) ophthalmic strips (Fluorets®, Bausch & Lomb). Corneal and conjunctival staining type was assessed on a 0 to 100 point scale where 0 represented no staining and 100 represented severe staining. Assesment of corneal and conjunctival staining was performed on all the participants who had the CIC procedure. Staining was

performed to assess the status of the ocular surface post CIC procedure. This data is not used in this thesis.

3.13 Analytical procedures

3.13.1 Total protein isolation from the CIC samples

Total protein isolation from the CIC samples was performed for ID # 1 to 86 (explained in detail in appendix D). CIC samples were placed on a glass plate with adhered cells facing up. 10 µL of Extraction Buffer (EB), containing 50mM Tris and 2% SDS, + Complete Protease Inhibitor Cocktail (PI) (Roche Diagnostics, 1X concentration) was applied to each membrane. The discs were cut up using a scalpel into 1- 2mm pieces, added to an eppendorf tube containing an additional 50µL of EB, boiled for 10 minutes, spun at 12,000 relative centrifugal force (VWR Mini Vortexer, VWR International, USA), and the supernatant was aliquoted and stored at -80º C.

3.13.2 Total protein determination

Total protein determination was performed using the DC Protein Assay Kit™ (BioRad, Mississauga, ON, Canada) (Cat # 500-0116) as per the manufacturer's instructions (appendix E). Tear samples and IC supernatant were diluted in distilled water. For sample preparation: 0.5µL of capillary tears; 5µL of eye wash or 5µL of IC supernatant was added to sufficient Milli-Q water to total 10 µL. All samples and standards were assayed in duplicate 5µL aliquots (explained in detail in appendix E). Absorbances were read at 750 nm on a Multiskan Microplate Spectrophotometer (Thermo Labsystems, Franklin, MA, USA) (Cat# 28010; Figure 3-16). Graphs were plotted from the standard readings. Using the standard linear regression equation, the amount of protein per well and then per µL of sample was calculated.

Figure 3-16: Multiskan Microplate Spectrphotometer

3.13.3 Quantification of individual lacrimal gland tear proteins (lipocalin and lysozyme)

3.13.3.1Electrophoresis and immunoblotting

Electrophoresis and immunoblotting techniques are explained in detail in appendix F. In brief, for the analysis of lipocalin, tear samples were diluted to final concentrations of 10 ng/µL (capillary tears) or 15 ng/µL (eye wash), 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 dithiolthreitol (DTT), 0.01% Bromophenol blue). For lysozyme analysis, tear samples were first diluted with Laemmil's buffer to 50 ng/µL (capillary tears) or 25 ng/µL (eye wash) and then to final concentrations of 25 ng/µL or 15 ng/µL (capillary and eye wash, respectively) with gel loading buffer (60 mM Tris (pH 6.8), 2% glycerol, 2% SDS, 0.01% bromophenol blue).

To facilitate quantitation of tear samples, standard curves were run on each gel. For lipocalin, this was a titration (5 to 30 ng/µL total protein) of pooled human tears collected from non dry-eyed volunteers and for lysozyme, a titration (1-10 ng/µL) 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; Figure 3-17). 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 µg total protein, whereas lysozyme data are expressed as µg per µg total protein.

Figure 3-17: Amersham Pharmacia Biotech PhastSystem™

3.13.3.2 Quantification of densitometric Data

The concentration of lysozyme and lipocalin in each sample was quantified by densitometric analysis using ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA, USA). The resultant densitometric peak volumes of the standards were graphed versus the appropriate protein concentrations producing a standard curve. Linear regression was performed and the equation of the line-of-best-fit was used to calculate the lysozyme/lipocalin concentration in the sample extracts. The densitometric volumes for the sample extracts were substituted into the equation:

$$\mathbf{x = (y - b)/m}$$

where y is the densitometric volume of the sample extract, m is the slope of the line calculated by linear regression, b is the y axis intercept as calculated by linear regression, and x is the concentration of purified lysozyme/ lipocalin. In many cases, the standard curve was more curved and the best-fit line was found to be a quadratic polynomial (SigmaPlot software V9.01) with equation:

$$\mathbf{y = y_o + ax + bx^2}$$

where y_o, a and b were calculated by the software and x is the densitometric volume of the sample extract. For the curved standard curves analyzed this way, r^2 values were always 0.98 or better with this method.

3.13.4 Detection of MUC 1 and 16 from tears and CIC samples

3.13.4.1Agarose gel electrophoresis and immunoblotting

Agarose gels (2% agarose (Cambrex Seakem 50150) (w,v), 2.5% glycerol (v/v) (EMD chemicals 4750) 1x gel buffer (v/v) (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS) (BioRad 162-0147) were poured one day before use. Gel cassettes of 16 cm by 18 cm were assembled according to manufacturer's directions. One volume of tears was mixed with one volume 2X non-reducing buffer (final concentration 25 mM Tris-HCl pH 6.8, 2% SDS). One and a half millimeter thick gels were run at 20 mA constant current per gel in 25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS, BioRad 162-0147. To facilitate quantitation of tear and IC samples, standard curves were run on each gel. Six micrograms of total protein for MUC1 and 4 ug for MUC16 were loaded. Once prepared, samples and standards were subjected to agarose gel electrophoresis. SE600 Vertical gel unit (GE Biosciences (Amersham) Cat. No. 80-6479-57) was used for electrophoresis, Figure 3-18.

This was followed by transfer on to nitrocellulose membrane (0.2 uM, 13.5 x 16.5 cm, BioRad 162-0147) using a vacuum blotter (Biorad 785, Cat. No. 165-5001) and 2 L of transfer buffer (600 mM sodium chloride, 60 mM sodium dihydrogen citrate (4x SSC), EMD 8310) for 2 hours. Blots were blocked with PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4, 0.5% Tween 20(v/v) and 0.1% BSA (bovine serum albumin, (Sigma A3059) (w/v) for 1 hour at room temperature. MUC 1 was identified using

antibody detection with mouse monoclonal anti-human MUC 1 antibody, DF3 (Signet Dedham, MA) (1:40) diluted in PBS – T solution overnight at 4°C.

Blots were rinsed several times in PBS-T and then incubated at room temperature in the goat anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc) secondary antibody (1:5000) diluted in PBS-T. MUC16 was identified by incubating the blot in monoclonal mouse antihuman MUC16 antibody, OC125, (DAKO, Glostrup, Denmark) (1:250) diluted in PBS –T overnight at 4C. Blots were rinsed several times in PBS-T and then incubated at room temperature in the goat anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc) secondary antibody (1:5000) diluted in PBS-T. Immunoreactivity was visualized by incubating with ECL-Plus™ chemiluminescent substrate. Optical densities of the resulting bands were quantified from digitized images collected 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 MUC 1 and MUC16 as described above for lysozyme and lipocalin.

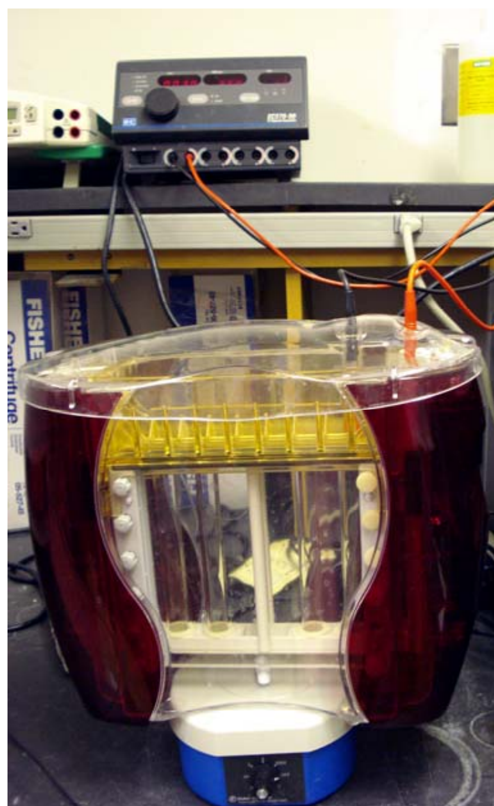

Figure 3-18: SE600 Vertical gel unit

3.13.5 Isolation of RNA from CIC samples

RNA was isolated from the CIC samples using commercially available RNeasy™ Mini Kit (Qiagen Cat # 74106) and the DNAse step was performed using the RNase-Free DNase Set™ (Qiagen Cat# 79254).

3.13.5.1Preparation of RNase-Free DNase

DNase stock solution was prepared before using the RNase-free DNase for the first time. The solid phase of the DNase (1500Kunitz units[14]) was dissolved in 550 µl of the RNase-free water provided. This was mixed gently by inverting the tube. This stock solution was aliquoted into samples of 22µL and stored in -20°C until further use.

3.13.5.2 RNA isolation using commercially available RNeasy Mini kit:

The RNA was isolated from the samples according to manufacturer's guidelines with several modifications (appendix G). Briefly, samples soaked in RLT buffer were vortexed and homogenized. One volume of 70% ethanol was added to each sample followed by a 15 second vortex. Samples were added to the RNeasy mini spin column and centrifuged. DNAse step was performed which included a 15 minute incubation period. This was followed by two RPE buffer washes and centrifugation at 8000g. RNeasy mini spin column was dried and placed in 2mL collection tubes. 40µL of RNAse free water was pipetted onto the column and centrifuged for 1 minute at 8000g to collect RNA samples.

3.13.6 Precautions and handling RNA

Great care was taken during conjunctival cell sample collection, during and after RNA isolation procedures, to avoid RNAse contamination and to maintain an RNAse-free environment. Nitrile gloves were always worn by the examiner during sample collection, during and after RNA isolation procedures to prevent RNAse contamination from skin, or from laboratory equipment. Gloves were changed frequently.

Disposable polypropylene tubes were used throughout the procedure. These tubes were purchased as RNAse-free and did not require subsequent treatment to inactivate RNAses. Disposable plugged pipette tips that were meant for RNA work were used for pipetting solutions. All laboratory glassware was cleaned with detergent, followed by thorough rinsing with MilliQ water and baked at 230°C for at least 9 hours. All solutions for RNA work were taken from stocks reserved for RNA work only. The lab bench surfaces and equipment (eg. centrifuge, pipette) were routinely treated with RNAase

ZAP™ solution (Ambion®, Foster City, CA, USA) to remove surface RNAse contamination. Instruments (flat and pointed forceps) used for performing impression cytology were sterilized at 230°C for 9 hours.

3.13.7 RNA quality and quantity assessment and cDNA synthesis

RNA quantity and quality was assessed by measuring the optical density using a Beckman DU530 Life Science UV/Visible Spectrophotometer (Beckman Coulter, Fullerton, CA; Figure 3-19), at 260nm and 280nm. Five microliters of sample was diluted in 95µL Milli-Q water. Background subtraction of the value obtained at 320 nm was also performed. cDNA was synthesized from 8µL 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 instruction.

Figure 3-19: Beckman DU530 Life Science UV/Visible Spectrophotometer

3.13.8 Real time qPCR

Relative expression of genes of interest was performed in multiplex PCR reactions containing target and endogenous control oligonucleotide primers in the presence of gene-specific dye-labeled Taqman probes (Table 3-2). Two microlitres of cDNA was used for amplification in a 50µL PCR reaction containing target (300 nM) and endogenous control (100 nM) oligonucleotide primers, control and target Taqman probes (100 nM), and Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Duplicate samples were used for analysis in a 7500 Real Time PCR System (Applied Biosystems; Figure 3-20).

Conditions used for amplification were as follows: 50°C for two 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. 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, Foster City, CA). A sample containing 0.25 pg of plasmid DNA with cloned target and endogenous fragments was used as a calibrator sample for each gene.

Figure 3-20: Applied Biosystems: 7500 Real Time PCR System

Table 3-2: Oligonucleotide primers and probes used for relative expression analysis

Gene	Forward Primer	Reverse Primer	Taqman Probe
MUC1	CTGGTCTGTGTTC TGGTTGC	CCACTGCTGGGTT TGTGTAA	6FAM-GAAAGAACTACGGGCA GCTG
MUC16	ACCCAGCTGCAG AACTTCA	GGTAGTAGCCTGG GCACTGT	6FAM-GCGGAAGAAGGAAGGA GAAT
GAPDH	GAAGGTGAAGGT CGGAGTCA	GACAAGCTTCCCG TTCTGAG	VIC-CAATGACCCCTTCATTG ACC

3.14 Averaging and pooling of clinical and biological data

The data collected from the right and left eye for NITUBT, PRT and subjective and objective bulbar conjunctival hyperemia values were averaged for subsequent analysis. There was no significant difference noted between these parameters within the two eyes.

The tear samples collected using disposable capillary tubes were pooled (section 3.6). Tear washes were pooled together and stored at -80°C until use (section 3.10).

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4 Assessment of Ocular Surface dryness Using Dry Eye Questionnaires in Postmenopausal Females

Srinivasan S, Joyce E, Senchyna M, Jones LW. Assessment of ocular surface dryness using dry eye questionnaires in postmenopausal females. In Submission (Optometry and Vision Science)

4.1 Abstract

Aim: To characterize symptoms of dry eye in a group of postmenopausal women (PMW) using the Allergan Ocular Surface Disease Index Questionnaire© (OSDI) and Indiana Dry Eye Questionnaire (DEQ).

Methods: Eighty two healthy PMW, who were non-contact lens wearers and not on hormone replacement therapy, completed two questionnaires. They were categorized as being symptomatic or asymptomatic of dry eye based on their response to the OSDI questionnaire. The participants also completed the DEQ, which has questions related to frequency of ocular surface symptoms and their diurnal intensity.

Results: OSDI responses revealed 43 symptomatic (mild = 16; moderate = 27) and 39 asymptomatic participants. The OSDI total score for the Non Dry Eye (NDE) and Dry Eye (DE) groups were significantly different (NDE =7.43 ± 7.71 vs DE = 24.87 ± 13.89; $p<0.001$). The sub scores for the DE group were also significantly greater than the NDE group ($p<0.001$). The DEQ scores showed that the DE group exhibited a higher

frequency and intensity of symptoms than the NDE group ($p<0.001$), which worsened as the day progressed ($p<0.001$).

Conclusions: Dry eye questionnaires can be used to separate PMW who demonstrate symptoms of ocular dryness and may be useful in treatment trials in dry eye. This also emphasizes the fact that in addition to clinical assessment, a careful history and the use of dry eye questionnaires are necessary for accurate dry eye diagnosis.

4.2 Introduction

Dry eye questionnaires are tools that are frequently used in clinical research to screen individuals for the diagnosis of dry eye. They are also employed in clinical practice to grade the severity of the disease or to assess the effects of various dry eye treatments.[1] Dry eye questionnaires are also used in population based studies and to study the natural history of disease. The epidemiology sub committee of the Dry Eye Workshop 2007 (DEWS) has reviewed and identified several questionnaires that were used previously,[2-14] either in randomized clinical trials or that have been tested or used in epidemiological studies.

When clinicians attempt to classify patients with signs and symptoms of dry eye, a wide battery of tests are available. Of these, a careful patient history and the use of dry eye questionnaires have been shown to be very useful in the diagnosis of dry eye. Many complain of dry eye symptoms that may precede clinically observed ocular surface changes.[15] In contrast, clinical signs may be observed by practitioners in the absence of patient reported symptoms of ocular dryness, and several studies have reported this lack of agreement between signs and symptoms of this disease.[16-18] The most recent definition proposed by the Dry Eye Workshop defines dry eye as a multifactorial ocular surface disease diagnosed by ***symptoms of discomfort*** and signs of visual disturbance, tear film instability and ocular surface damage, accompanied by increased osmolarity of the tear film and ocular surface inflammation.[19]

Studies have shown that clinicians rely to a large degree on their case history, with the patient's input regarded as an important tool to diagnose and categorize dry eye patients.[15, 20, 21] Hence, symptoms of dry eye are a key factor in the diagnosis and

treatment of dry eye. It has been reported that the incidence and prevalence of dry eye increases with aging.[14, 22-24] Studies have shown that the number of women affected with dry eye disease is higher than that found in men.[25-28] Menopause can play an important role in causing dry eye symptoms.[1, 29, 30] The Women's Health Study [8] estimated that 3.2 million women aged over 50 suffer from moderate to severe forms of dry eye.[1]

A variety of questionnaires are available for the evaluation of symptoms, which can aid in the diagnosis of dry eye.[1-14, 31] Of these, the Allergan Ocular Surface Disease Index (OSDI) © questionnaire (appendix A) is a validated questionnaire,[7] which includes relatively few questions and is more easily applied than many of the other longer, more complex questionnaires that are available. The questionnaire originally had 40 items, which was later reduced to a simpler 12 item questionnaire, to provide a rapid assessment of symptoms.[7]

End of day ocular surface dryness is a frequently reported complaint from both those subjects who exhibit signs and symptoms of dry eye and contact lens wearers.[9] Many of these patients use ocular lubricants to alleviate their symptoms, particularly at the end of the day. One of the questionnaires that has been reported to assess ocular surface symptoms of dryness and diurnal fluctuations in symptoms, especially in mild to moderate dry eye patients, is the Indiana Dry Eye Questionnaire (DEQ).[9, 32] The DEQ consists of a total of 23 questions (appendix C). It includes questions about frequency and the diurnal severity of common ocular surface symptoms reported by symptomatic dry eye individuals. In addition to questions related to ocular symptoms, questions on how much these symptoms interfere with day to day activities are also present.

The purpose of this study was to characterize symptoms of dry eye in a group of postmenopausal women (PMW) using two different dry eye questionnaires, namely the Allergan OSDI questionnaire and the Indiana DEQ.

4.3 Materials and Methods

The protocol for this study was approved by the Office of Research Ethics at the University of Waterloo. Study participants were recruited at the Centre for Contact Lens Research, within the School of Optometry. A case history and complete ocular surface examination was performed to determine participant eligibility. Informed consent was obtained from all participants, after all the procedures had been explained. Participants who were on Hormone Replacement Therapy (HRT) were excluded, due to the confounding results present in the literature on whether HRT is protective or exacerbates dry eye symptoms and signs.[33, 34] Non-contact lens wearers (ceased lens wear at least 1 year before participating in the study) and candidates with systemic disease, or using any systemic or topical medications that may have affected ocular health, were also excluded from the study. For the purpose of this study, “postmenopausal” was defined as no menses for at least one year, not associated with hysterectomy. This research study adhered to the tenets of the Declaration of Helsinki.

Eighty two healthy PMW greater than 50 years of age were recruited. Participants completed the Allergan Ocular Surface Disease Index© (OSDI)[7] questionnaire and the Indiana Dry Eye Questionnaire (DEQ)[9, 32] and were categorized as being symptomatic or asymptomatic of dry eye based on their response to the OSDI questionnaire. The administration and scoring system for the OSDI has been described in detail elsewhere[7]

Broadly, the OSDI is a questionnaire that includes 12 questions, which are subdivided into three groups: ocular symptoms, vision related functions and environmental factors. The OSDI scoring is based on a 0-100 scale, with the highest score representing greater disability. The OSDI questionnaire is graded on a scale from 0 to 4, where 0 indicates "none of the time"; 1, "some of the time"; 2, "half of the time"; 3, "most of the time"; 4, "all of the time". The following formula is used to find the total OSDI score: OSDI = [(sum of scores for all questions answered) × 100] / [(total number of questions answered) × 4]. An OSDI score of 0-12 represents NDE; an OSDI score of 13-22 is categorized as mild DE and an OSDI score of 23-32 represents moderate dry eye.[7, 35]

The questions in the Indiana DEQ related to ocular symptoms included discomfort, dryness, visual changes, soreness and irritation, grittiness and scratchiness, foreign body sensation, burning and stinging, light sensitivity, and itching. The questionnaire also included questions about age, how much ocular symptoms affected daily activities, questions concerning computer use, history of contact lens wear, use of systemic and ocular medications, allergies, self-assessment of whether subjects thought they had dry eye, and whether subjects had been previously diagnosed as having dry eye. However, this questionnaire does not have a specific formula to compute the scores, as described above for the OSDI questionnaire.

4.4 Statistical analysis

Statistical analysis was performed using Statistica Ver7.1 (StatSoft Inc., Tulsa, OK, USA). Graphs were plotted using GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA). OSDI scores and sub-scores between the two groups were compared using a Mann Whitney U test.

DEQ analysis: A Mann Whitney U test was performed to compare the frequency of scores (Questions 4 to 12), issues relating to the frequency of the subjects' eyes feeling "bothered" (Question 13a), the number of hours of computer use (Questions 14a and b), and dryness elsewhere in the body (Question 17). Diurnal shifts (morning to evening) of the scores within each group was compared using Friedman analysis of variance and across the groups (non dry eyed group vs dry eyed group) were compared using a Mann Whitney U test.

A Chi-square test was performed to compare DEQ allergy, medication use and artificial tear use (Questions 15 and 16a and 20). A Chi-square test was also performed for questions pertaining to self diagnosis and doctor's diagnosis of dry eye (Questions 18a and 19). Correlations between OSDI and DEQ were undertaken using a Spearman rank correlation. A p value of less than 0.05 was considered to be statistically significant.

4.5 Results

4.5.1 OSDI classification

The mean age (mean ± SD) of the NDE subjects (n=39) was 59.7 ± 6.6 years and 64.1 ± 9.2 years for the DE participants (n=43). The OSDI total score was significantly different between the two groups (NDE = 7.43 ± 7.71 vs DE = 24.87 ± 13.89; $p<0.001$). The symptomatic group of participants consisted of 16 mild and 27 moderate dry-eyed patients (there were no subjects who were categorized as being "severe"). All three OSDI sub-scores were also significantly different between the symptomatic and asymptomatic groups, with higher scores for all three subcategories for the dry-eyed subjects ($p<0.001$; summarized in Table 4-1).

Due to the length and number of questions (23 questions) in the DEQ, the mild and moderate dry eye groups were combined together as the "dry eyed" group in the rest of the results section.

Table 4-1: Summary of Ocular Surface Disease Index© scores

OSDI Score	Overall			Subcategory				
	NDE (n=39)	**DE (n=43)**	**p (α =0.05) (NDE vs DE)**	**Mild DE (n=17)**	**Moderate DE (n=26)**	**p (α =0.05) (NDE vs Mild)**	**p (α =0.05) (NDE vs Moderate)**	**p (α =0.05) (Mild vs Moderate)**
Total Score	7.43 ± 7.71	24.87 ± 13.89	**<0.001***	18.37 ± 9.29	28.31 ± 13.02	**<0.001***	**<0.001***	**0.01***
Ocular Symptoms	7.56 ± 8.42	23.98 ± 19.21	**<0.001***	17.35 ± 13.59	26.73 ± 24.49	**0.001***	**<0.001***	0.10
Vision Related Functions	7.05 ± 12.18	17.19±16.42	**0.002***	8.33 ± 7.21	22.20 ± 21.42	0.68	**<0.001***	**0.005***
Environmental Triggers	8.22 ± 12.29	34.09 ± 25.97	**<0.001***	30.39 ± 21.02	36.86 ± 27.21	**<0.001***	**<0.001***	0.43

4.5.2 DEQ responses

4.5.2.1 Frequency and intensity of ocular symptoms

Figures 4-1and 4-2 demonstrate the responses to questions related to the frequency of nine ocular symptoms by NDE and DE PMW. Over 55% of the dry eyed PMW demonstrated symptoms of frequent to constant discomfort. 47% complained of frequent to constant dryness symptoms. Other symptoms such as blurry vision, soreness and irritation, grittiness and scratchiness, foreign body sensation, burning and stinging, light sensitivity, and itching ranged from 11 to 37%. This is markedly different to the control subjects, who reported frequent to constant discomfort and dryness at only 5% and 0%, respectively. There was a significant difference between the asymptomatic and symptomatic PMW reporting all symptoms ($p < 0.05$, Mann Whitney U test).

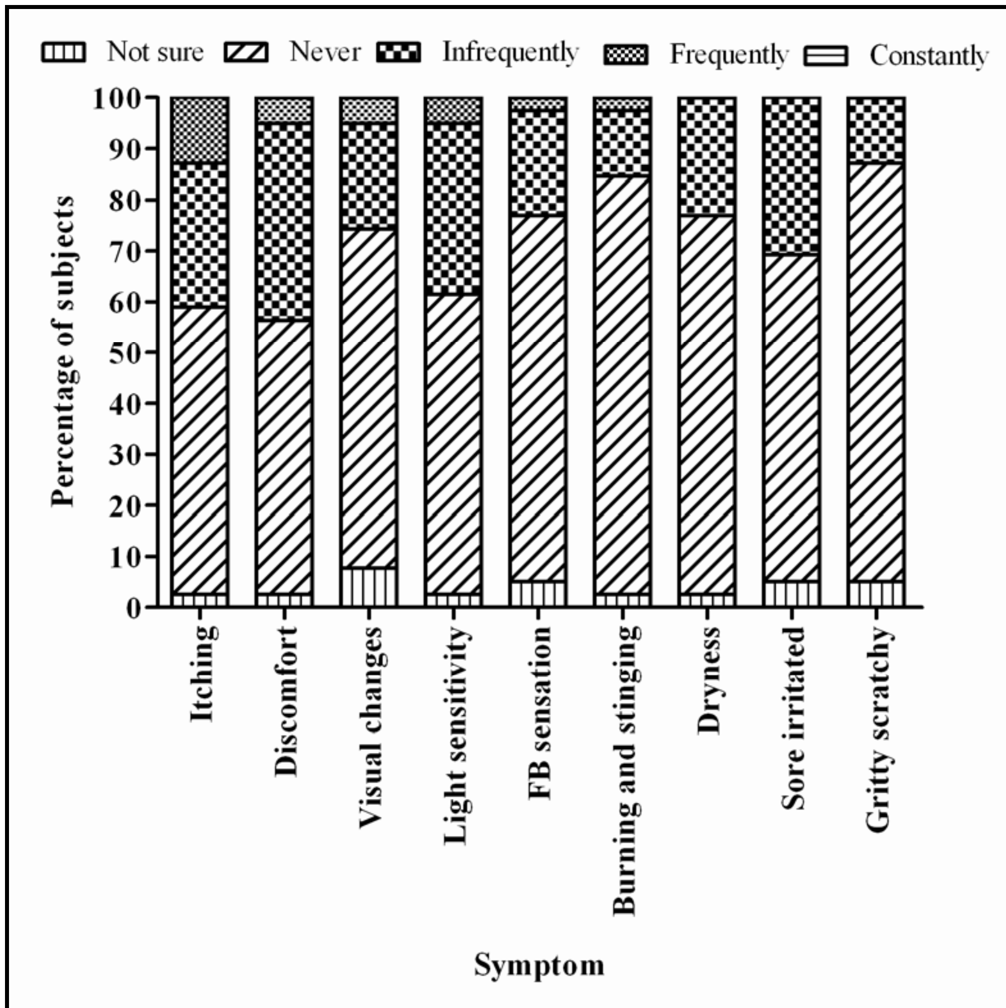

Figure 4-1: Frequency of symptoms in NDE participants using DEQ

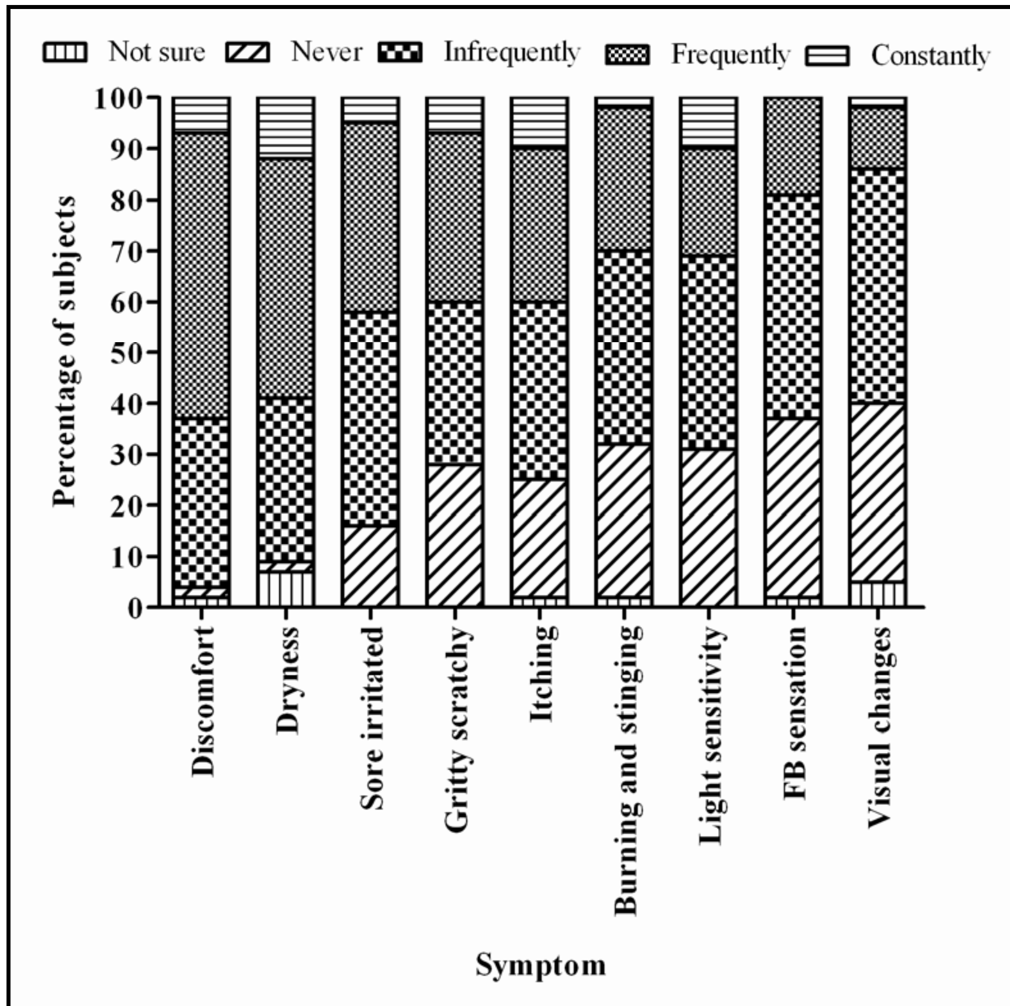

Figure 4-2: Frequency of symptoms in DE participants using DEQ

4.5.2.2 Diurnal variation of ocular symptoms

Figures 4-3 and 4-4 demonstrate the diurnal variation in symptoms in the non dry eyed and the dry eyed group respectively. When morning and evening scores were compared within each group (with the exception of "soreness"; p=0.02), no change in symptoms were reported over the course of the day (p>0.05) within the non dry eyed group. However, the dry eyed group showed significant differences, with increasing symptoms of dryness towards the end of the day for all the symptoms evaluated

($p<0.005$), except for “light sensitivity” ($p=0.052$). When symptoms at the various points in time were compared between groups, Mann-Whitney U test showed significant differences between the two groups for the majority of symptoms, including comfort, dryness, soreness and irritation, grittiness and scratchiness, burning and stinging symptoms ($p<0.001$). The dry eyed group showed higher scores than the non dry eyed group throughout the day as shown in Figures 4-5 and 4-6. The rest of the symptoms (blurry vision, foreign body sensation and itching) showed significant differences only in the evening. Light sensitivity was significantly different between the two groups only for the mid-day measurement ($p<0.001$)

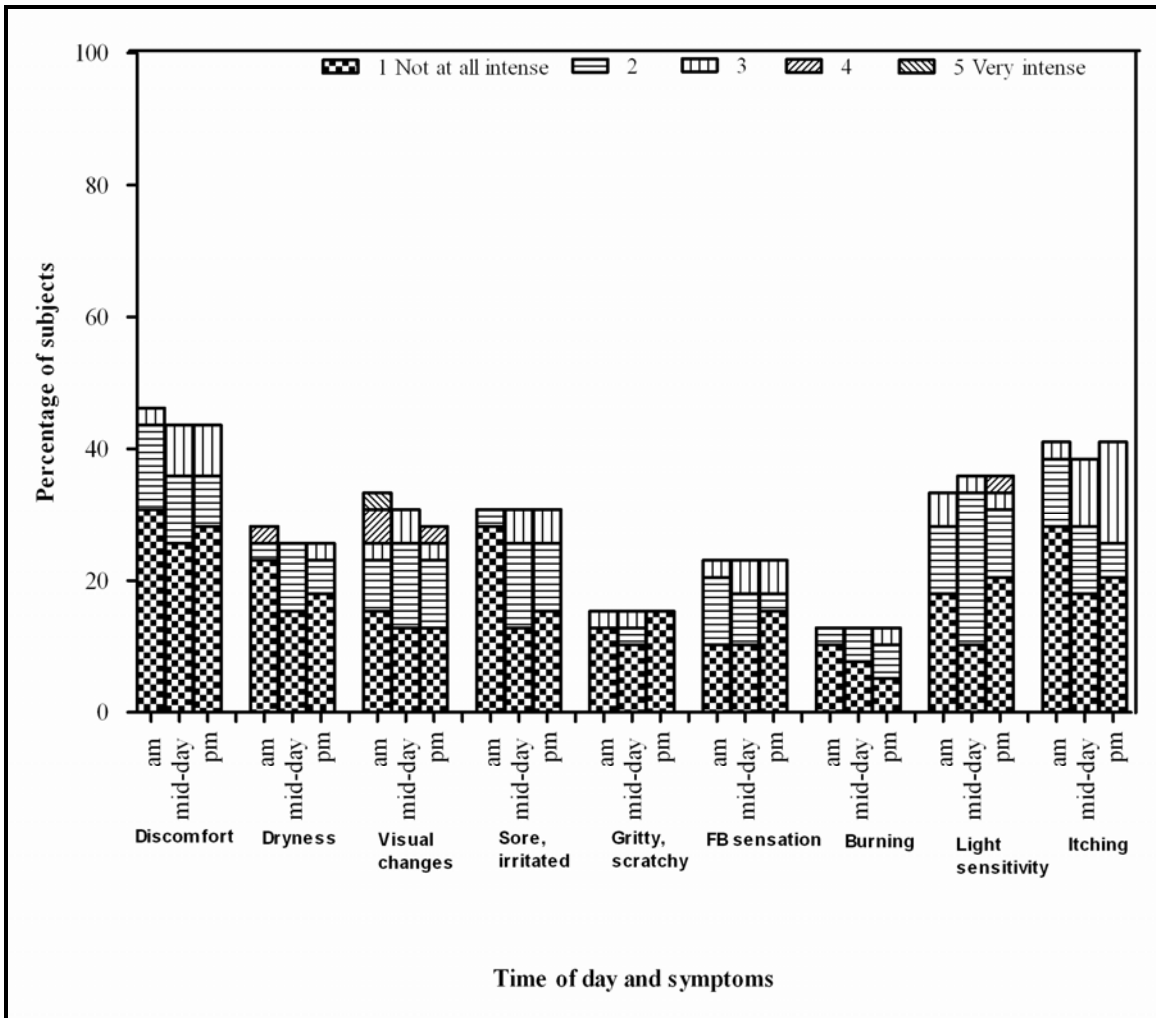

Figure 4-3: Diurnal intensity of symptoms of dry eye in NDE participants

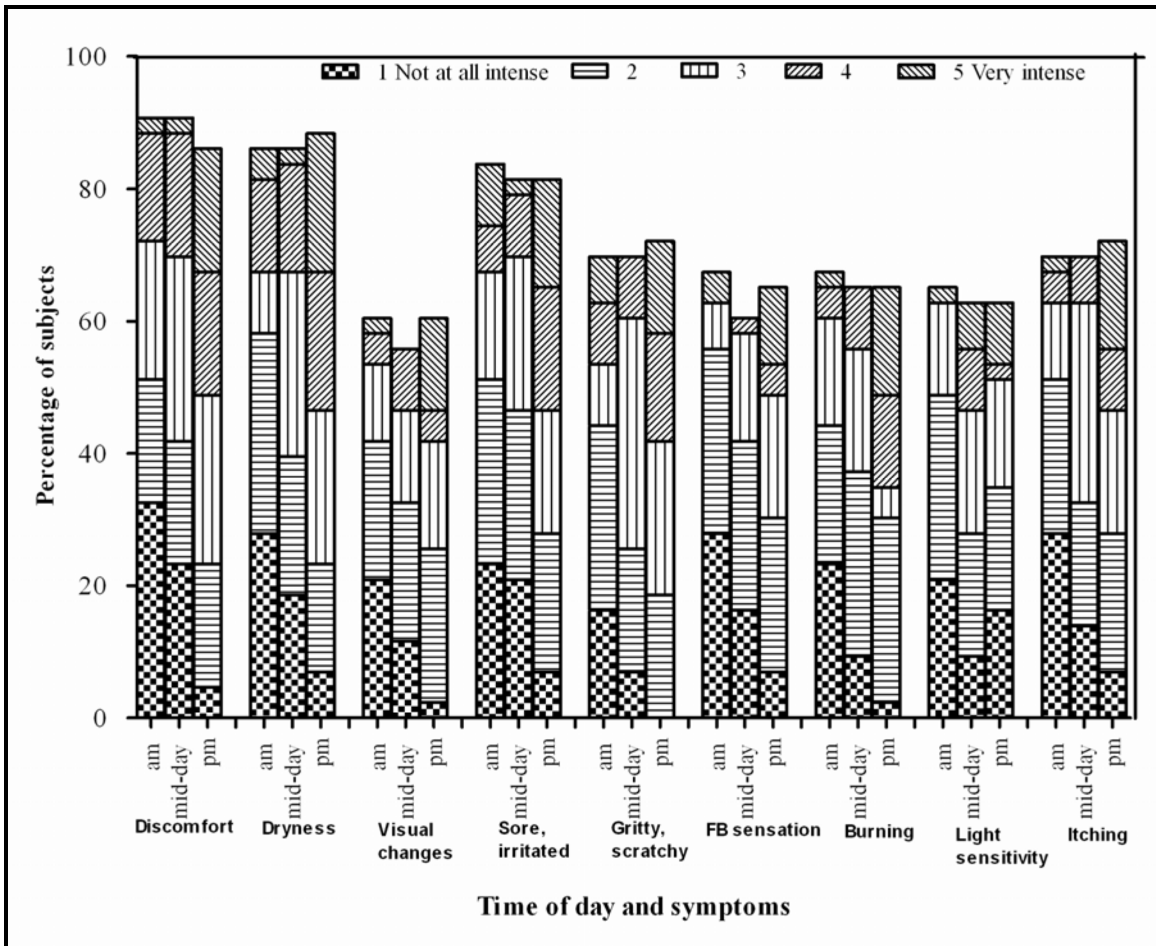

Figure 4-4: Diurnal intensity of symptoms of dry eye in DE participants

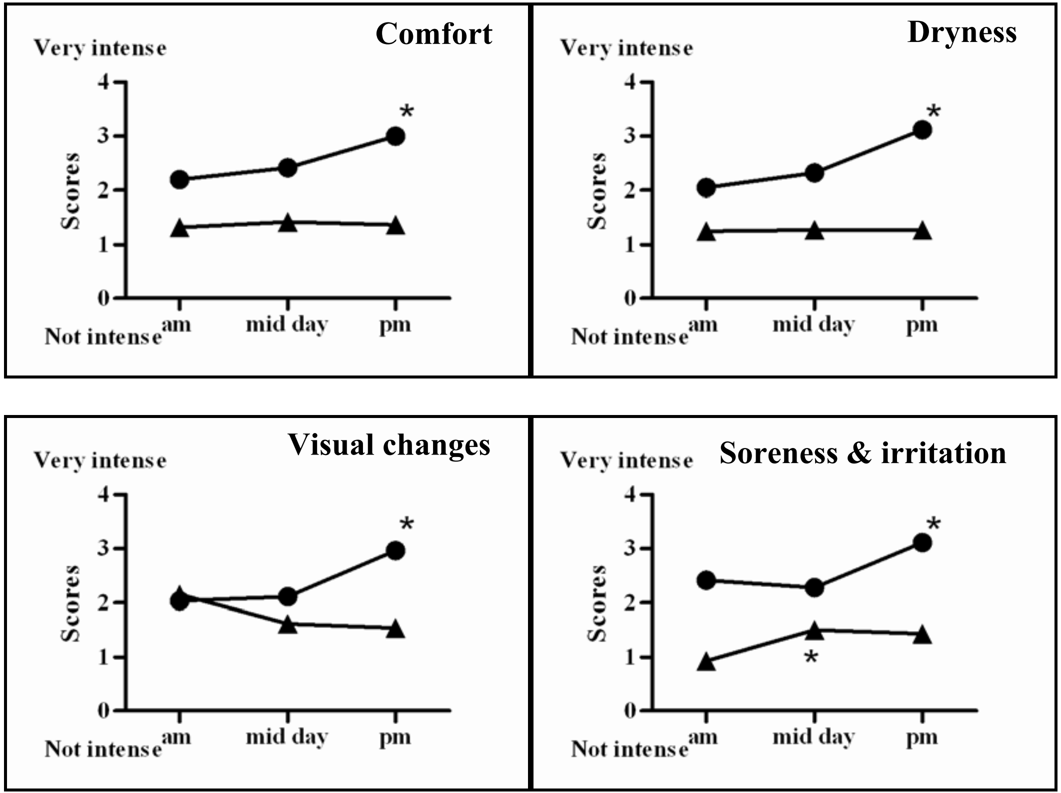

Figure 4-5: Morning (am) middle of the day (mid day) and evening (pm) symptom mean intensity score reported by dry eyed and non dry eyed PMW

● represents dry eyed participants and ▲ represents non dry eyed participants. * represents statistically significant difference between groups over time.

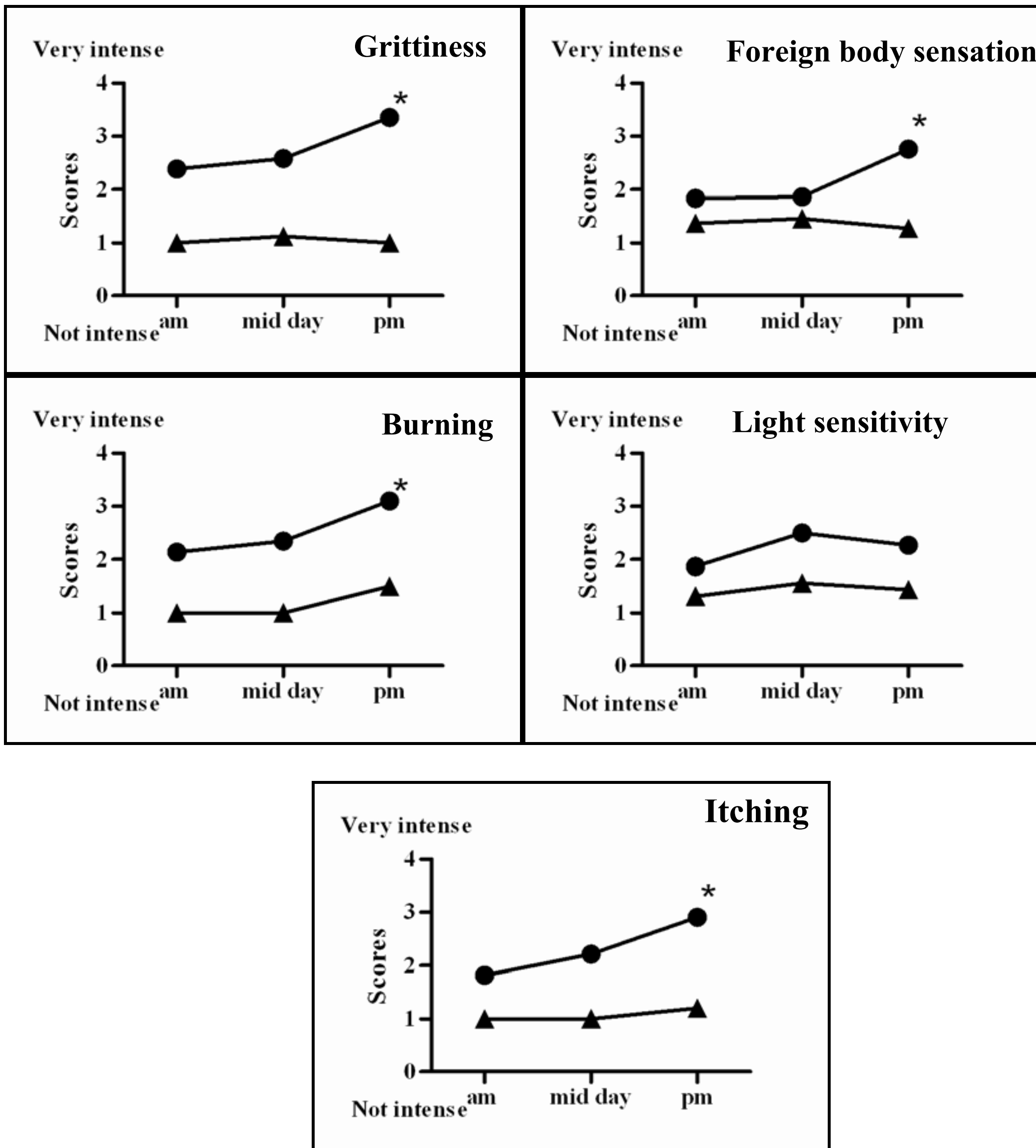

Figure 4-6: Morning (am) middle of the day (mid day) and evening (pm) symptom mean intensity score reported by dry eyed and non dry eyed PMW

● represents dry eyed participants and ▲ represents non dry eyed participants. * represents statistically significant difference between groups over time.

The participants were also asked to comment on the frequency at which their eyes felt bothered during the previous week that made them stop their work. Over 32% of the symptomatic PMW reported "frequently" and 30% of the women complained of only

“infrequent” symptoms. About 19% of the subjects complained of their eyes being bothered several times during the week. The percentage of participants who reported their “eyes being bothered daily” and “several times daily” were 14% and 12% respectively. 92% of the asymptomatic women reported that their eyes were not bothered.

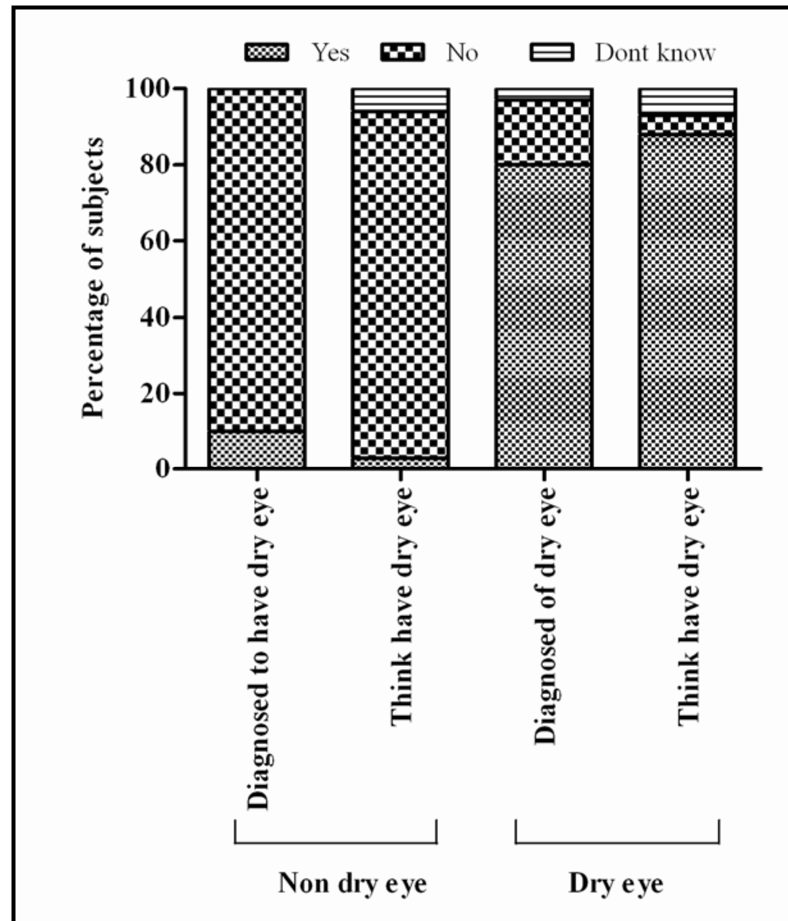

Figure 4-7: Frequency of self diagnosis of dry eye vs doctors diagnosis of dry eye

When the participants were asked about how often they experienced dryness of the mouth, nose or vagina in the past month, 44% of the DE PMW reported dryness that was present “daily.” In contrast, the percentage of NDE PMW who reported dryness was 15%. This was statistically significantly different ($p>0.001$). About 80% of the DE participants and 5% of the NDE participants reported that they had previously been told

that they had dry eyes by their clinician (Figures 4-7). Three percent of the DE group did not know if they had been diagnosed. When participants were asked “do you think you have dry eye(s)?”, 89% of the DE women and 2% of the NDE group reported “yes.” Seven percent of the DE and 5% of the NDE participants were not sure (Figures 4-7).

4.5.2.3 Computer usage

In this study, computer usage was slightly greater among the asymptomatic controls, but the difference was not significant among groups ($p = 0.145$, Mann Whitney U test). About 38% of the NDE and 53% of the DE post menopausal women did not use computers for work related reasons. On average, NDE subjects reported 2.6 hours of computer use on normal working day and 1.2 hours on a nonworking day, DE subjects reported 1.7 hours of workday computer 1.4 hours on a leisure basis.

4.5.2.4 Medication and allergies

Allergies and medication usage is listed in Tables 4-2 and 4-3. The percentage of subjects reporting allergies was very similar among the two groups. Current usage of medication and allergy medication (if any) was also similar among both groups ($p>0.05$, Chi-square test).

Table 4-2: Response to questions on medication usage in DEQ

Medications	NDE		DE	
	YES (%)	NO (%)	YES %)	NO (%)
Thyroid	13	87	19	81
Blood pressure	28	72	23	77
Diabetes	0	100	0	100
Diuretic	18	82	7	93
Acutane	0	100	0	100
Heart condition	3	97	5	95
Depression	5	95	12	88
Ulcer	0	100	7	93
Oral contraceptives	0	100	0	100

Table 4-3: Response to questions related to allergies in DEQ

Allergies	NDE			DE		
	YES (%)	NO (%)	DON'T KNOW (%)	YES %)	NO (%)	DON'T KNOW (%)
Seasonal	33	64	3	33	60	7
Skin	5	95	0	9	86	5
Asthma	5	95	0	14	81	5
Animals	15	85	0	23	68	9
Pollen and mold	21	77	2	19	67	14
food	5	92	3	21	74	5
Affecting your eyes	23	74	3	19	58	23
Allergies to Contact lens solutions	0	90	10	2	74	24
Eye drops	0	90	10	0	81	19

4.5.2.5 Dry eye treatments

Question 18b in the questionnaire targeted the effectiveness of dry eye treatments, such as artificial tears, warm compresses, lid scrubs and punctal plugs. About 30% of the dry eyed participants reported responses midway between “no help at all” and a “complete cure,” as shown in Table 4-4. Fewer subjects reported using lid scrubs, warm compresses and punctal plugs, with similar relief reported. Sixty two percent of DE women on an average instilled artificial tears four times a day (range = 0 to 8 times) and spent $9 (range = $0 to $30) a month on the purchase of artificial tears. Few of the participants used samples of artificial tear drops, if available.

Table 4-4: Relief provided by dry eye treatment

DE	No help at all (% subjects)	Mid way between no help and complete cure (% subjects)			Complete “cure”(% subjects)	Do not use (% subjects)
Artificial tears	2	23	30	16	5	23
Warm compress or lid cleaning	2	2	9	5	0	81
Punctal plugs or cauterization	0	0	2	0	0	98

4.5.2.6 Correlations

Spearman correlation analysis between OSDI total score and DEQ for symptom comparison showed weak but significant associations (R=0.45 to 0.65; $p<0.001$) as shown in table 4-5. Similar results were observed for Spearman correlation analysis between OSDI sub scores and DEQ showing significant correlations (Ocular symptom: R=0.49 to0.72; $p<0.001$; Vision related functions: R=0.24 to 0.52; $p<0.05$; Environmental triggers: R=0.33 to 0.50; $p<0.001$).

Table 4-5: Correlation between OSDI total score and frequency of symptoms of dry eye (Questions 4 to 12 from DEQ)

OSDI	Spearman R	p value
Discomfort	0.65	<0.0001
Dryness	0.59	<0.0001
Blurry vision	0.46	<0.0001
Soreness	0.58	<0.0001
Grittiness & scratchiness	0.53	<0.0001
Foreign body sensation	0.45	<0.0001
Burning and stinging	0.57	<0.0001
Light sensitivity	0.52	<0.0001
Itching	0.50	<0.0001

4.6 Discussion

In this study the symptoms of dry eye were characterised in a group of PMW using validated questionnaires, namely the Allergan Ocular Surface Disease Index© (OSDI) Questionnaire and the Indiana Dry Eye Questionnaire (DEQ).

As pointed out by Begley et al,[9] clinicians often rely on symptoms alone to arrive at a diagnosis and treatment plan for dry eyed patients, without any sign of visible tissue damage.[9] Population-based studies of the elderly have shown that the proportion identified by any clinical tests that reveal visible signs of tissue damage is far less than that revealed by patient reported dry eye symptoms.[23]

Our attempt to classify participants as being symptomatic or asymptomatic of dry eye using an OSDI questionnaire was demonstrable, with statistically significant differences between the OSDI total score and sub scores (NDE =7.43 ± 7.71 vs DE = 24.87 ± 13.89; $p<0.001$). Following grouping of the subjects based on the results from the OSDI questionnaire, subsequent completion and analysis of the DEQ revealed that >55% of the dry eyed PMW demonstrated symptoms of frequent to constant discomfort and 47% complained of frequent to constant dryness symptoms. This is consistent with other studies performed previously using the DEQ in a non-Sjogren's dry eyed group.[9, 10, 36] Other symptoms experienced by the dry eyed group, such as soreness, grittiness, visual changes and itchy eyes, showed a range of 11- 37% for frequent to constant symptoms. The diurnal shift in the intensity of symptoms in the dry eyed PMW was towards the higher side in the evening, as seen in Figures 4-4, 4-5 and 4-6. This is in agreement with previous studies using the DEQ, with a similar diurnal variation in Sjogren's-related dry

eye, as well as contact lens wearers and non-Sjogren's dry eye, with Sjogren's related dry eye showing the maximum increase at the end of the day.[9, 10, 36]

Many non dry-eyed PMW reported "never or infrequent" symptoms, but they rarely reported "frequent to constant" symptoms (Figure 4-1), nor did they report very "intense symptoms" (Figure 4-3). Some of the non dry-eyed PMW showed a trend of increasing symptoms of burning and itching towards the end of the day, but this was still not significant. This emphasizes the effect that the environment,[36] workplace, and humidity may have on the tear film.[37]

Over 32% of the symptomatic PMW reported the presence of symptoms "frequently", which caused them to cease their activities. About 19% of the subjects complained that their eyes were bothered significantly several times during the week. The percentage of participants who reported their "eyes being bothered daily" and "several times daily" were 14% and 12% respectively. Even though 60% of the dry-eyed PMW used artificial tears regularly, only 5% of them reported that the drops provided a "complete cure", suggesting that the 95% of PMW who use artificial tears experience only partial relief.

The DEQ comprises a variety of symptom-based questions for a variety of ocular surface symptoms and their changing intensity over the course of the day. The Contact Lens DEQ, that was developed to study the dryness responses in contact lens wearers,[38] has a method/formula to obtain a total symptom score. Even though the DEQ is a long and extensive questionnaire and is one of the very few that can target any potential diurnal variation in symptoms, it lacks a scoring method to compute a final symptom

score, unlike the McMonnies[4] or OSDI[7] questionnaires. This remains one of the major disadvantages of using the DEQ in a clinical environment.

Schiffman et al[7] studied 109 cases with dry eye disease and 30 control subjects. The scores on both the OSDI and two other questionnaires (McMonnies Dry Eye Questionnaire and National Eye Institute Visual Functioning Questionnaire-NEI-VFQ) were compared, and a significant correlation was observed.[7] Nichols et al have also shown significant correlations between the OSDI and NEI-VFQ.[31] Similar results were demonstrated by Vitale et al.[39]

In this study, when an attempt to correlate the OSDI (total score) versus the DEQ frequency scores was made, there was a significant correlation (R values 0.45 to 0.65; $p<0.001$). The range of R values noticed in Table 4-5 may be due to the fact that the OSDI total score is a combination of questions related to ocular symptoms, vision related functions and environmental triggers. The end point is a combination of scores from different symptoms. This compares with the DEQ, which has 9 individual questions on symptoms targeted on dryness symptoms on a categorical scale. This emphasizes the fact that both surveys can be used for grouping individuals as dry eyed and non-dry eyed. They have certain questions in common; however, each questionnaire has its uniqueness. OSDI can be used to separate PMW who demonstrate symptoms of ocular dryness. In addition to a variety of dry eye symptom questions, the DEQ is a useful tool to study the diurnal variations in dry eye symptoms.

Currently there is no internationally accepted criterion for the diagnosis of dry eye disease, but the OSDI is a standardized tool to evaluate symptoms, and can easily be performed and used to symptomatically diagnose dry eye. It is suggested that symptoms

assessment should be included as an important part of the diagnostic assessment and management of dry eye patients, to determine their response to treatment.[9]

4.7 Conclusion

Participants categorized as being symptomatic of dry eye using OSDI also demonstrated higher frequency and worsening dry eye symptoms towards the end of the day. Although dry eye disease lacks a strong correlation between objective tests and subjective symptoms, symptoms are an important aspect of dry eye disease. The need to understand the role of each individual questionnaire still exists.

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5 Clinical signs and symptoms in postmenopausal females with symptoms of dry eye

Srinivasan S, Joyce E, Jones LW. Clinical signs and symptoms in postmenopausal females with symptoms of dry eye. Ophthalmic and Physiological Optics. In press. Permission obtained from Blackwell Publishing Ltd.

5.1 Abstract

Aim: To characterize clinical signs and symptoms in a group of postmenopausal women (PMW) who present with and without symptoms of dry eye.

Methods: Eighty three healthy PMW were categorized as being symptomatic or asymptomatic of dry eye based on their response to the Allergan Ocular Surface Disease Index© (OSDI) questionnaire. Non invasive tear breakup time (NITBUT) was evaluated using the ALCON Eyemap®. Tear volume was assessed using the Phenol Red Thread (PRT) test and bulbar conjunctival hyperemia was measured using objective and subjective methods.

Results: The total OSDI score (TOS) and sub scores for the (n= 39) Non Dry Eye (NDE) and (n= 44) Dry Eye (DE) groups were significantly different (TOS: NDE = 7.43 ± 7.71 vs DE = 24.87 ± 13.89; $p<0.001$). The DE group exhibited a shorter NITBUT (5.3 ± 1.7 vs 7.0 ± 2.7 secs; $p=0.0012$). Tear volume was lower for the DE group (19.3 ± 5.1mm vs. 16.3 ± 5.6mm; $p=0.031$). Bulbar hyperemia was higher in the DE group for both

subjective techniques (48.4 ± 10.0 vs 40.6 ± 10.4; $p=0.0011$) and objective ($u' = 0.285 \pm 0.006$ vs. 0.282 ± 0.006; $p=0.005$).

Conclusions: OSDI can be used to separate PMW who demonstrate clinical signs of ocular dryness. PMW with dry eye symptoms demonstrate shorter NITBUT, lower tear volume and increased bulbar conjunctival hyperemia than those who have no symptoms.

5.2 Introduction

The incidence and prevalence of dry eye increases with increasing age[1] and the number of women affected with dry eye disease is higher than that found in men.[1-3] The Women's Health Study[1] estimated that 3.2 million women aged over 50 suffer from moderate to severe forms of dry eye.[4]

A reduction in sex hormones, such as androgens, occurs in both males and females with increasing age.[5] The hormonal changes that accompany menopause can play an important role in the production of dry eye symptoms,[4, 6, 7] and these symptoms may precede clinically observed ocular surface changes.[8] In contrast, clinical signs may be observed by practitioners in the absence of patient reported symptoms of ocular dryness,[8, 9] and several studies have reported this clear lack of agreement between the signs and symptoms that occur in patients with dry eye disease.[9-12]

The most recent definition proposed by the Dry Eye Workshop[13] defines dry eye as a "multifactorial ocular surface disease diagnosed by symptoms of discomfort and signs of visual disturbance, tear film instability and ocular surface damage, accompanied by increased osmolarity of the tear film and ocular surface inflammation".[13]

When clinicians attempt to classify patients with signs and symptoms of dry eye, a wide battery of tests are available.[14] Of these, a careful patient history and the use of validated dry eye questionnaires have been shown to be very useful in the diagnosis of dry eye.[10, 15-20] In addition, assessments of tear stability and tear volume have also shown differences between those patients presenting with and without dryness symptoms.[11, 14, 21-24] Given that the most recent definition of dry eye suggests that ocular surface inflammation is also involved,[13] then assessment of ocular surface hyperemia (redness),

which may act as a surrogate of mild inflammation, is also a variable worthy of study. However, to-date this latter issue of ocular surface hyperemia has not attracted much attention in the diagnosis of dry eye, despite the fact that it is a common complaint of those with irritated and uncomfortable eyes.[10, 17, 25, 26]

To-date, the majority of studies investigating ocular dryness symptoms and/or signs in postmenopausal women (PMW) have focused on the impact of hormone replacement therapy (HRT) or other drugs associated with controlling complications associated with cessation of menses.[27-35] The purpose of this study was to characterize a variety of clinical signs and symptoms in asymptomatic and symptomatic dry eyed PMW not on HRT, and to attempt to correlate these symptoms and signs.

5.3 Materials and Methods

The protocol for this study was approved by the Office of Research Ethics at the University of Waterloo. Study participants were recruited at the Centre for Contact Lens Research at the School of Optometry. Informed consent was obtained from all participants after all the procedures had been explained. A case history and complete ocular surface examination was performed to determine participant eligibility. Participants who were currently taking HRT were excluded, due to the confounding results present in the literature on whether HRT is protective or exacerbates dry eye symptoms and signs.[30, 31] Non-contact lens wearers and candidates with any form of systemic disease or those who were taking any systemic or topical medications were also excluded from the study. For the purpose of this study, "postmenopausal" was defined as no menses for at least one year, not associated with hysterectomy. This research study adhered to the tenets of the Declaration of Helsinki.

Eighty three healthy PMW who were greater than 50 years of age were recruited in this study. Participants completed the Allergan Ocular Surface Disease Index© (OSDI)[36, 37] and were categorized as being symptomatic or asymptomatic of dry eye based on their response to OSDI. The administration and scoring system for the OSDI has been described in detail elsewhere.[36, 37] Broadly, the OSDI is a questionnaire that includes 12 questions, which are subdivided into three groups: ocular symptoms, vision related functions and environmental factors. The OSDI scoring is based on a 0-100 scale, with the highest score representing greater disability. The OSDI questionnaire is graded on a scale from 0 to 4, where 0 indicates none of the time; 1, some of the time; 2, half of the time; 3, most of the time; and 4, all of the time. The following formula is used to find

the total OSDI score: OSDI = [(sum of scores for all questions answered) × 100] / [(total number of questions answered) × 4]. The OSDI scores and sub-scores for the two groups were then recorded and compared. An OSDI score of 0-12 represents NDE; an OSDI score of 13-22 is categorized as mild DE and an OSDI score of 23-32 represents moderate dry eye.[36, 37]

One observer, who was masked with respect to whether the subjects were considered dry-eyed or not, was responsible for collecting all the data. Clinical tests were undertaken in the following order. Tear stability was assessed by performing a non invasive tear breakup time (NITBUT) evaluation using the ALCON Eyemap® model EH-290 topography system (ALCON, Inc., Forth Worth, Texas, USA). The keratoscope unit produces concentric rings of light, which are reflected off the cornea and imaged in the CCD camera. Participants were comfortably seated with their head supported by a forehead and chin rest and looked at a fixation light at the centre of the concentric rings of light. Participants were asked to blink 3 times before each measurement was taken. NITBUT was measured by measuring the time taken for distortions or discontinuities to appear in the reflected image of the concentric ring pattern. The time (in seconds) for the tear-film to rupture (and thus distort the rings) was measured using a stopwatch, to the nearest 0.1 of a second. Three measurements were taken in each eye and the average of these was used for analysis purposes.

Tear volume was assessed using the Phenol Red Thread (PRT) test (ZONE-QUICK, Showa Yakuhin Kako Co., Ltd. Tokyo, Japan). The PRT was placed at a point approximately 1/3 of the distance from the lateral canthus of the lower eyelid with the eye in primary position, as previously reported.[38] The lower lid was pulled down gently,

and the folded 3mm portion of the thread was placed on the palpebral conjunctiva at the position specified. Each eye was tested with the subjects' eyes open for 15 seconds. During the test the patients were instructed to look straight ahead and blink normally. After 15 seconds, the lower lid was gently pulled down, and the thread was gently removed with an upward motion. Care was taken to pull the eyelid down before removal of the thread to avoid discomfort. The length of the color change on the thread, which indicates the wetting length, was measured immediately in millimeters from the very tip regardless of the fold. No topical anesthetic was used. A stop watch was used to measure the time.

Bulbar conjunctival hyperemia was assessed using both subjective and objective methods. The slit lamp was used to subjectively grade the level of temporal bulbar conjunctival hyperemia using a modified CCLRU scale, which uses a 0-100 scale (0 – negligible, 25 - trace 50 - mild 75 – moderate, 100 – severe).[39-41] The subjects were comfortably seated at the slit lamp and the position of gaze was directed to allow grading of hyperemia on the temporal bulbar conjunctiva of both eyes. Hyperemia was assessed under diffuse white light with 16X magnification. Mean value (left and right eye) was recorded for use in subsequent analysis.

Objective bulbar redness was undertaken using a SpectraScan PR650© Spectrophotometer (Photo Research Inc, Chatsworth, CA) under controlled illumination. It is a table top device that determines measurements of luminance and chromaticity by measuring the absolute intensity at each wavelength and then calculating the equivalent CIE u' value.[39, 42] The subject sat at the photometer and looked at a fixation light to their left or to the right, such that the temporal conjunctiva was aligned with the instrument.

The examiner looked through the eye piece and positioned the black measuring spot (approximately 19.63 mm^2) of the photometer approximately 2 mm from the temporal limbus on the temporal bulbar conjunctiva. The spectrophotometer was turned on just before the measurement and turned off immediately after, to ensure that the ocular surface temperature did not increase and changes in ocular surface hyperemia were minimized. Redness was measured three times on both eyes. Mean value (left and right eye) was recorded for use in subsequent analysis.

Corneal and conjunctival staining could not be performed as a part of this study due to the nature of the study design, which included collection of tears and conjunctival impression cytology samples for subsequent biomarker analysis (reported in later chapters). The addition of fluorescein and/or other vital dyes would affect the subsequent analysis of the biomarkers and was thus not undertaken.

5.4 Statistical analysis

Statistical analysis was performed using Statistica Ver7.1 (StatSoft Inc., Tulsa, OK, USA). NITBUT, PRT values, subjective and objective bulbar hyperemia and OSDI scores were compared using Mann Whitney U test. Correlations were undertaken using a Spearman rank correlation. A p value of less than 0.05 was considered to be statistically significant.

5.5 Results

All data are expressed as means ± standard deviation. The mean age of the participants was 63.6 ± 9.4 yrs in the symptomatic group (n=44) and 59.5 ± 6.6 yrs in the asymptomatic group (n=39). Of the 83 participants enrolled, only one participant reported

severe dry eye symptoms and was thus excluded from this analysis, resulting in 43 participants in the dry eye group (mild = 16, moderate = 27).

Table 5-1 details the OSDI total score and sub scores for both groups, with the symptomatic dry eye (DE) group being subdivided by their severity of symptoms. The total and sub-scores for the DE group overall were significantly greater than the asymptomatic non dry eye (NDE) group ($p<0.001$).

Table 5-1: Summary of Ocular Surface Disease Index© scores

OSDI Score	Overall			Subcategory				
	NDE (n=39)	DE (n=43)	p (α =0.05) (NDE vs DE)	Mild DE (n=16)	Moderate DE (n=27)	p (α =0.05) (NDE vs Mild)	p (α =0.05) (NDE vs Moderate)	p (α =0.05) (Mild vs Moderate)
Total Score	7.43 ± 7.71	24.87 ± 13.89	**<0.001***	18.37 ± 9.29	28.31 ± 13.02	**<0.001***	**<0.001***	**0.01***
Ocular Symptoms	7.56 ± 8.42	23.98 ± 19.21	**<0.001***	17.35 ± 13.59	26.73 ± 24.49	**0.002***	**<0.001***	0.10
Vision Related Functions	7.05 ± 12.18	17.19 ± 16.42	**0.002***	8.33 ± 7.21	22.20 ± 21.42	0.11	**<0.001***	**0.003***
Environmental Triggers	8.22 ± 12.29	34.09 ± 25.97	**<0.001***	30.39 ± 21.02	36.86 ± 27.21	**<0.001***	**<0.001***	0.68

A summary of the clinical measures is reported in Table 5-2. The DE group exhibited a shorter NITBUT and lower tear volume than the NDE group. The DE group had slightly increased levels of bulbar hyperemia assessed both subjectively and objectively, when compared with the NDE group. When the DE group was broken down into mild and moderate subgroups, no significant differences were found in the NITBUT, PRT and conjunctival bulbar redness between NDE and the mild DE group. Comparison of clinical data between mild and moderate sub groups also showed no statistically significant differences. However, all the clinical measures showed a significant difference between the NDE and moderate DE group ($p<0.05$), as described in Table 5-2.

Table 5-2: Summary of clinical measures in sub groups

Clinical measures	Overall			Subcategory				
	NDE	DE	P (α =0.05) (NDE vs DE)	Mild DE	Moderate DE	p (α =0.05) (NDE vs Mild)	p (α =0.05) (NDE vs Moderate)	p (α =0.05) (Mild vs Moderate)
NITBUT (seconds)	7.0 ± 2.7	5.3 ± 1.7	**0.0012***	5.7 ± 1.9	5.1 ± 1.2	0.105	**0.002***	0.31
PRT (mm)	19.3 ± 5.1	16.3 ± 5.6	**0.031***	16.8 ± 6.0	16.4 ± 6.6	0.14	**0.03***	0.96
Conjunctival hyperemia (subjective measure)	40.6 ± 10.4	48.4 ± 10.0	**0.0011***	45.4 ± 7.3	50.0 ± 5.0	0.058	**0.001***	0.221
Conjunctival hyperemia (u') (objective measure)	0.282 ± .006	0.285 ± 0.006	**0.005***	0.284 ± 0.005	0.286 ± 0.005	0.126	**0.013***	0.221

Spearman correlations between the clinical signs and total OSDI scores were weak (0.341 to -0.293). Statistically significant correlations ($p<0.05$) were found for three of the clinical measures (NITBUT $r = -0.293$; subjective redness $r = 0.341$; objective redness $r = 0.278$). The exception to this was PRT, which showed no statistically significant correlation ($r = -0.117$; $p>0.05$). Correlations between subjective and objective redness were also significant ($r = 0.660$; $p< 0.001$). Correlations between clinical signs with OSDI sub scores were also weak (0.10 to 0.282).

5.6 Discussion

In this study, we attempted to characterize and compare clinical signs and symptoms in two groups of PMW. A validated questionnaire was used as a tool to categorize these females into dry eyed and non dry eyed individuals and NITBUT, PRT and bulbar conjunctival redness measurements were undertaken and compared in these two groups.

A very recent study[34] has evaluated the effect of HRT on dry eye in PMW, comparing the results to forty age-matched untreated women, who acted as controls. To our knowledge, the study we are reporting here is the first that has studied clinical signs (including subjective and objective redness measurements) and symptoms in PMW who are not on HRT. HRT usage has shown confounding results[29, 32] in terms of exacerbation of dry eye disease.[34] Use of estrogen alone has shown an increase in the prevalence of dry eye,[28] as opposed to estrogen combined with progesterone.[28, 31, 33] This is a major reason why we decided to exclude subjects who were taking HRT from this study.

The OSDI questionnaire measures the severity of dry eye disease based on symptoms, functional changes and environmental triggers.[36, 37] Analysis of Table 5-1 shows the utility of using OSDI to categorise PMW with and without symptoms of dry eye and the ability of OSDI to subcategorise those with mild and moderate symptoms. Using this questionnaire, the majority of the symptomatic group was categorized as having "moderate" symptoms. Based on severity of symptoms, the symptomatic group with moderate dry eye symptoms showed significantly higher total scores and sub-scores in relation to NDE participants. The participants who had mild dry eye symptoms also showed higher scores when compared to NDE. The exception to this trend was vision related functions, which showed no statistically significant difference.

A stable and structurally intact tear film is essential for a smooth ocular surface. One of the tear stability tests suggested in the DEWS diagnostic methodology[14] and earlier studies[43-45] is non invasive tear film break up time (NITBUT). This test is repeatable,[43-45] sensitive[14, 46] and provides an accurate measurement to differentiate between symptomatic and asymptomatic dry eyed individuals.[21, 26 14] Our data supports the fact that NITBUT is a clinical test that can differentiate between two populations with and without symptoms of dry eye, as shown in Table 5-2. The results from our study clearly demonstrate that the dry eye group had poor tear film stability. This could be due to multiple factors, including a deficiency or abnormality of any of the components of the tear film.[47] Aging is associated with significant sex-related alterations in the polar and neutral lipid profiles of human meibomian gland secretions.[5] Studies have shown that the meibomian gland is an androgen target organ and deficiency of androgen may promote meibomian gland dysfunction and tear film instability and dry eye, and androgen

deficiency may be an important etiologic factor in the pathogenesis of evaporative dry eye in women with Sjogrens syndrome.[5, 48-51] Our values are comparable to a recently published study.[34]

The Schirmer Test has long been considered the “gold standard” method to evaluate tear film volume and production. However, it has some significant disadvantages due to its ability to stimulate tear production. In order to overcome the inherent difficulties and disadvantages of the Schirmer test[21, 52], the less “invasive” phenol red thread (PRT) test was developed.[53] The PRT test time is significantly lower than that required for the Schirmer test, at only 15 seconds, and the discomfort level is minimal, resulting in less reflex tear production[54] and this was the test used in our study. The exact parameter of the tears that is measured by the PRT test is open to debate. Sakamoto and co-workers suggested that PRT measures the volume of the residual tear film in the inferior conjunctival sac.[55] Other studies have shown that the PRT test estimates the presence and magnitude of the tear volume, rather than production rate itself.[56] A recent study by Tomlinson and colleagues[57] reported that the PRT test demonstrates different absorption characteristics of the thread, based on the biophysics or composition of tears, which allows the differentiation between dry eye and normal subjects. Studies that have compared the Schirmers test and PRT have shown a weak agreement between the tests.[11, 24, 43, 54] This is possibly because each test measures different aspects of the tear film.[54] Studies have also shown that the agreement between the symptoms as measured by the validated questionnaire and either test were poor for all the cutoff values used.[54, 58, 59] Regardless of what PRT test measure, the results from this

study demonstrated significantly lower PRT values in the DE group in comparison with the NDE group, as shown in Table 5-2.

Recently, we have reported poor tear ferning patterns and increased osmolality in tears collected from PMW with mild to moderate forms of dry eye (chapter 6),[12] emphasizing the fact that the composition of the tear film does appear to be different in DE and NDE PMW. An evaluation of our PRT data does indicate that the values reported are higher than those anticipated, with the values for DE group being higher than the cut off 9mm[53] and 10mm[43] that have been proposed by previous authors. However, the suggestion of a cut off value comes from a study that included a population with a wide age range and both genders,[53, 55] which is quite different to this study, which includes only older female subjects. To our knowledge, no PRT cut off value has been proposed for older age groups alone. As one would expect, age typically results in reduced tear volume.[60, 61] However, a recent study has shown that older individuals tend to exhibit a higher tear meniscus height (and hence tear volume), due to age-related constriction of the puncta, resulting in limited drainage of tears from the ocular surface.[62] Thus, it is entirely feasible that our PRT data for our older group of PMW is appropriate, potentially due, in part, to punctal stenosis. However, this requires further investigation.

The recent acceptance of the fact that many dry eye conditions are related to increased inflammation[13] led us to measure the levels of bulbar conjunctival redness of our subjects, which is one of the most common symptoms reported by individuals who are symptomatic of dry eye[25, 43] and could be considered to be a potential surrogate of ocular inflammation. The results from this study show increased levels of bulbar conjunctival hyperemia in the symptomatic group, by both objective and subjective

methods (Table 5-2). The two methods of assessing bulbar redness were strongly correlated ($r = 0.660$; $p< 0.001$), demonstrating the utility of using objective redness assessments. These results corroborate others from our group from a different patient population[39] and show that objective redness assessments could be useful in multi-site clinical studies, where subjective redness assessments could exhibit significant inter-observer variability. One potential reason for increased bulbar hyperemia could be sub-clinical inflammation. This could be driven by multiple factors, including hormones, changes to the composition or quality of the tear film, environmental effects or inflammatory mediators (for example, cytokines) in the tear film. Further study is required to investigate and understand the physiology and mechanisms driving the increased hyperemia in dry eyed PMW.

5.7 Conclusion

PMW who are categorized as being symptomatic of dry eye show variable symptoms of ocular irritation when compared with a group of asymptomatic subjects. Participants symptomatic of dry eye exhibited shorter NITBUT and PRT values than an asymptomatic control group. In addition, greater levels of bulbar conjunctival hyperemia were observed (when measured both objectively and subjectively) in the symptomatic PMW, emphasizing the link between ocular surface redness and dryness. Correlations between most of the clinical signs measured and symptoms were weak, yet statistically significant.

Dry eye questionnaires such as OSDI can be used as an effective tool in categorizing subjects based on their severity of symptoms. PMW present a significant group of subjects with symptoms and signs of dry eye. They represent an ideal group of

individuals to study dry eye treatment and new therapies, including anti-inflammatory and decongestive agents. The majority of the subjects in the dry eyed group reported moderate symptoms of dryness and further studies are required to investigate more severe dry eye groups and also understand the changes to the ocular surface at the cellular level.

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6 Tear osmolality and ferning patterns in postmenopausal women

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6.1 Abstract

Purpose: To compare tear osmolality and ferning patterns in postmenopausal women (PMW) with and without dry eye symptoms.

Methods: Thirty-seven healthy PMW (>50 years of age), not on hormone replacement therapy, were categorized as being symptomatic or asymptomatic of dry eye based on their responses to an Allergan "Single-Item Score Dry Eye Questionnaire" (SIDEQ). They subsequently completed the Allergan "Ocular Surface Disease Index" (OSDI) questionnaire. Tear samples were collected from participants to evaluate osmolality and ferning patterns. A novel freezing point depression osmometer (Advanced Instruments Inc., Model 3100 Tear Osmometer), was used to measure the osmolality of the tear film. The tear ferning test was performed and evaluated for the quality of ferning based on the Rolando grading system.

Results: SIDEQ responses revealed 21 symptomatic and 16 asymptomatic participants. The OSDI total score was 6.5 +/- 5.9 for the non-dry-eyed (NDE) group and 25.7 +/- 12.4 for the dry-eyed (DE) group. The subscores for the DE group were significantly greater than the NDE group ($p < 0.001$). Osmolality values in DE individuals were significantly different from NDE (328.1 +/- 20.8 vs. 315.1 +/- 11.3 mOsm/kg; $p = 0.02$). Fifty percent of the DE participants showed type II ferning patterns and 29% of the DE participants showed type III ferning patterns, whereas the NDE participants showed either type I (44%) or II (66%) ferning patterns. There was a significant difference between the DE and NDE participants for the ferning patterns ($p = 0.019$). There was no significant correlation between tear osmolality and tear ferning (DE: $r = 0.12$; $p > 0.05$, NDE: $r = -0.17$; $p > 0.05$).

Conclusions: Osmolality in mild and moderately DE PMW is higher than in NDE PMW and tear ferning is a rapid, simple, noninvasive laboratory procedure that indicates altered tear quality in PMW with symptoms of dry eye.

6.2 Introduction

Dry eye can be broadly defined as any tear film anomaly that can interfere with normal ocular surface physiology.[1] There is an increased incidence and prevalence of dry eye among the elderly,[2] especially women aged 50 years and over,[3] which may affect their quality of life.[4] Menopause plays an important role in causing symptoms of dry eye syndrome (DES).[5, 6] To accurately diagnose patients with DES requires a suitable combination of patient symptoms and clinical signs.[7] The use of different dry eye questionnaires and a careful patient history have proved to be very useful in the assessment of dry eye.[8] However, several reports show that a poor correlation exists between the symptoms and signs of dry eye.[9-13]

A clinical test that has been suggested as being highly diagnostic involves measuring tear film osmolality.[14] This is often considered a "gold standard" in the evaluation of subjects with dry eye,[14-16] due to the hypertonic tear film found in dry eyed individuals.[1, 17-20] A hypertonic tear film causes ocular surface damage and may lead to discomfort.[1, 18, 19] Osmolality is a function of tear secretion, drainage, absorption and evaporation and can be regarded as a single parameter of tear film dynamics.[14] Even though tear osmolality has been considered the "gold standard" for diagnosing DES, it is not widely used clinically due to the lack of available equipment and the fact that most osmometers require a large volume of tears (typically 5-10μl),[8, 21] which limits its use in many dry eye subjects, particularly those with severe disease.[21, 22]

Measuring tear film osmolality is often undertaken using a freezing point depression method,[14] typically using the Clifton instrument.[16-20, 23, 24] However, this

instrument requires considerable expertise, is time consuming and the equipment is difficult to maintain.[16, 21, 22] Recently, two new osmometers have become available that use micro-volumes (<1μl) that make them ideally suited for use in dry eye research.[25, 26] We have recently reported on the use of a micro-volume freezing point depression instrument (Model 3100 Tear Osmometer; Advanced Instruments, Inc. Norwood, MA, USA), which is rapid and easy to use.[26]

Another test that has been reported to differ between dry-eyed and non dry-eyed individuals is the Tear Ferning Test (TFT). Tear fluid, when dried on a microscope slide, produces a specific "ferning pattern", which is believed to be due to an interaction between various electrolytes in the tears and macro-molecules such as proteins.[27] An increased salt concentration in tears (as occurs in hyperosmotic tears) and other changes in tear composition may cause an alteration in the ferning patterns observed.[28] Studies have shown that an alteration in tear ferning patterns occurs in dry eyed individuals and subjects aged over 40,[29] with a tendency for "less" ferning to be observed.

The purpose of this study was to determine tear osmolality and tear ferning patterns in a group of postmenopausal women (PMW) with and without symptoms of dry eye and to determine if any correlation between these tests exists.

6.3 Materials and Methods

The protocol for this study was approved by the Office of Research Ethics at the University of Waterloo. Study participants were recruited at the Centre for Contact Lens Research, School of Optometry. A case history and complete ocular surface examination was performed to determine participant eligibility. Informed consent was obtained from all participants after all the procedures had been explained. We excluded participants who were on Hormone Replacement Therapy (HRT) due to the confounding results present in the literature on whether HRT is protective or exacerbates dry eye symptoms and signs.[30, 31] Contact lens wearers and candidates with any major systemic disease or using any systemic or topical medications that may affect ocular health were also excluded from the study.

Thirty seven healthy PMW over 50 years of age were recruited in this study. Participants were categorized as being symptomatic or asymptomatic of dry eye based on their responses to a Single-Item Score Dry Eye Questionnaire (SIDEQ).[32] The SIDEQ self assessment questionnaire[32] assessed the subjects' ocular discomfort due to symptom of dryness on a 0-4 scale, ranging from "none" to "severe". Participants who reported "none" or "trace" symptoms were grouped in the non-dry eyed (NDE) group and the remainder were grouped into the dry eyed (DE) group. They subsequently completed the Allergan Ocular Surface Disease Index©[33] (OSDI) questionnaire. The administration and scoring system for the OSDI questionnaire has been described in detail elsewhere.[33] Broadly, the OSDI scoring is based on a 0-100 scale, with the highest score representing greater disability.[33] The OSDI scores for the two groups (which were derived using the SIDEQ questionnaire) were then recorded and compared.

6.3.1 Osmometry

Tear samples were collected from all participants between 9 AM and 11 AM to evaluate osmolality and ferning patterns. Approximately 2 µl of tears were collected from the inferior tear meniscus of the right eye of each subject using a single-use, graduated, disposable, sterile, smoothly polished, fine glass capillary tube (Wiretol-Micropipettes, Drummond Scientific Co., Broomall, PA, USA), as carefully as possible to reduce reflex tearing, at a slit lamp biomicroscope. The time taken to collect the sample was less than one minute, with most dry-eyed participants typically taking longer than the non dry-eyed subjects. This sample was then carefully transferred to an eppendorf tube for use in both the osmometer and the ferning test. Firstly, approximately 0.5 µl of these tears were transferred to a single-use, disposable polycarbonate capillary tube to load the sample into a novel freezing point depression osmometer (Model 3100 Tear Osmometer; Advanced Instruments, Inc. Norwood, MA, USA). Figure 6-1 represents typical crystal patterns before and during the freezing procedure. Tear samples varied in their time to freeze and the crystal patterns following freezing varied widely between participants.

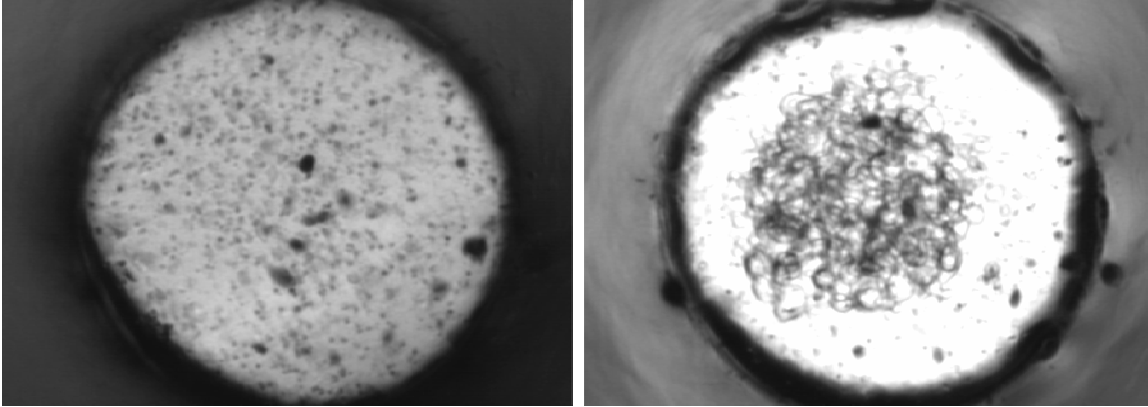

Figure 6-1: Tear film samples in the Model 3100 Tear Osmometer

The image on the left represents tear samples before freezing and the image on the right represents tear samples during the melting phase

6.3.2 Tear ferning

This method of collection allows a standard volume of tears to be collected and has also shown to result in reproducible ferning patterns.[34] Approximately 0.5 ul of tears was carefully pipetted out from the eppendorf and TFT was performed by dropping 0.5µl of the tear sample onto a clean glass microscope slide, which was allowed to dry at room temperature and evaluated at 10X magnification on a light microscope. The quality of ferning observed was based on the Rolando grading system,[35] which grades the ferning patterns from grades 1 (abundant ferning) through to grade 4 (no ferns). Photographs were taken immediately after drying to avoid misinterpretation in grading due to alterations in the ferning patterns, which may arise due to humidity and temperature changes in the environment.[27]

6.3.3 Statistical analysis

Statistical analysis was performed using Statistica Ver7.1 (StatSoft Inc., Tulsa, OK, USA). Differences in ferning patterns between the groups were determined using the Mann Whitney U test and osmolality values and OSDI scores were compared using a unpaired Student-t test. Correlations were undertaken using a Spearman rank correlation. A p value of less than 0.05 was considered to be statistically significant.

6.4 Results

The SIDEQ responses revealed 21 symptomatic (66.3 ± 9.8 yrs) and 16 asymptomatic (58.7 ± 5.7 yrs) participants. Figure 6-2 reports the total and sub-scores for the OSDI questionnaire. The OSDI total score was significantly different between the two groups (NDE 6.5 ± 5.9 vs DE 25.7 ± 12.4; $p<0.001$). All three OSDI sub-scores were also significantly different between the symptomatic and asymptomatic groups, with higher scores for all three subcategories for the dry-eyed subjects ($p<0.001$; see Figure 6-2).

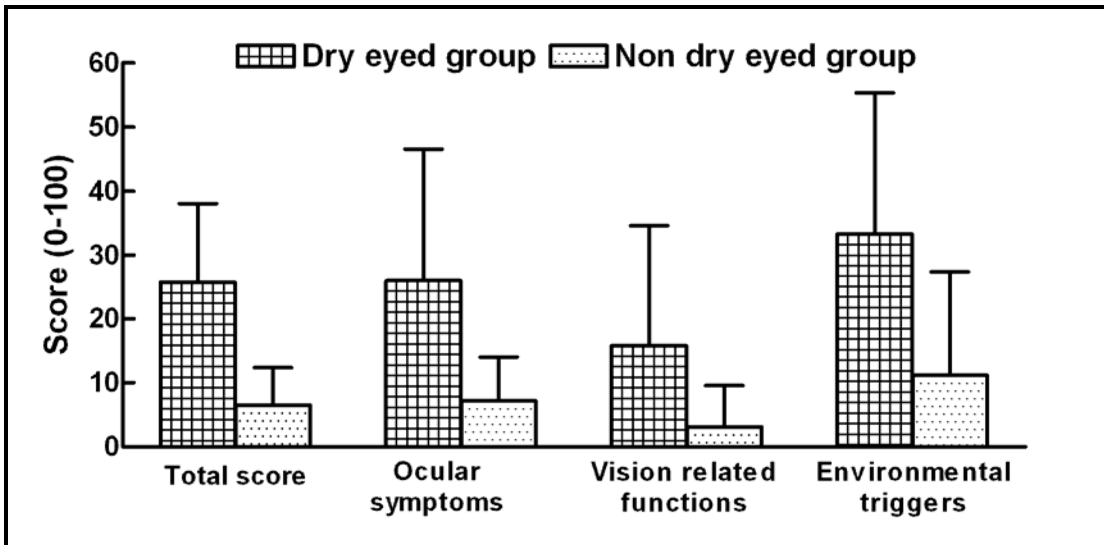

Figure 6-2: OSDI Questionnaire: Graph representing the total score, ocular symptoms, vision related, and environmental factors

The OSDI total score was significantly different between the two groups (NDE 6.5 ± 5.9 vs DE 25.7 ± 12.4; p<0.001. The error bars represents standard deviation) All three OSDI sub-scores were also significantly different between the symptomatic and asymptomatic groups, with higher scores for all three subcategories for the dry-eyed subjects (p<0.001).

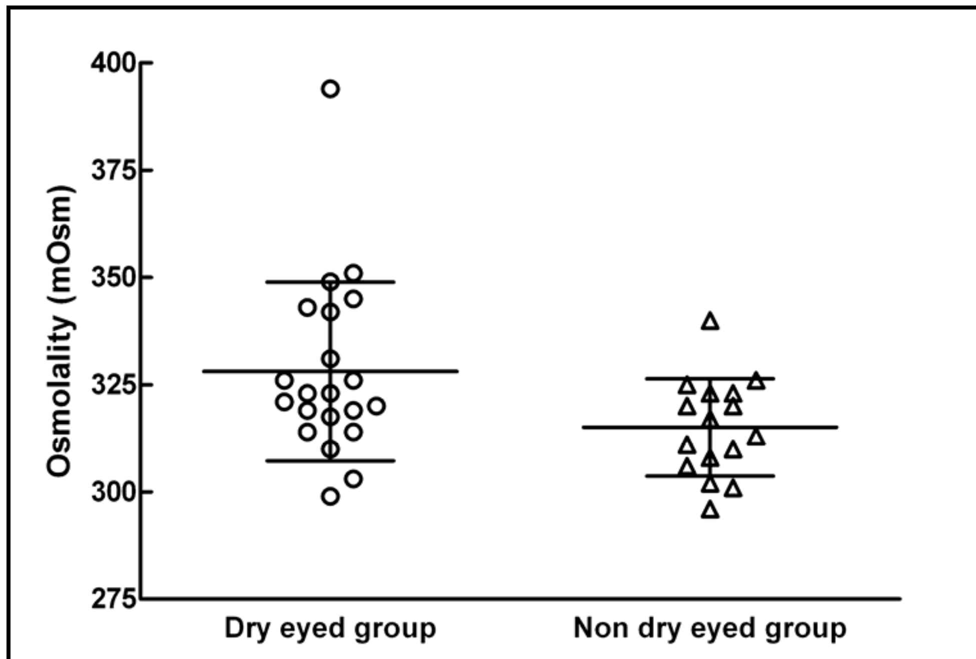

Figure 6-3: Osmolality in dry eyed (DE) and non dry eyed (NDE) participants

The mean osmolality values in the DE group was higher than that in the NDE group (328.1 ± 20.8 vs 315.1 ± 11.3 mOsm; p=0.02. The error bars represents standard deviation)

When ferning patterns were graded using the Rolando grading system, the majority of the symptomatic candidates showed type II ferning patterns (50%), while all of the asymptomatic participants showed either type I (44%) or type II (56%) ferning patterns (p=0.019) (Table 6-1). Typical ferning patterns seen are shown in Figures 6-4a-d.

Table 6-1: Represents the percentage distribution of dry eyed and non dry eyed participants showing ferning patterns (grade I to IV)

Ferning Grade	% of dry eyed participants showing ferning pattern	% of non-dry eyed participants showing ferning pattern
I	14	44
II	50	56
III	29	0
IV	7	0

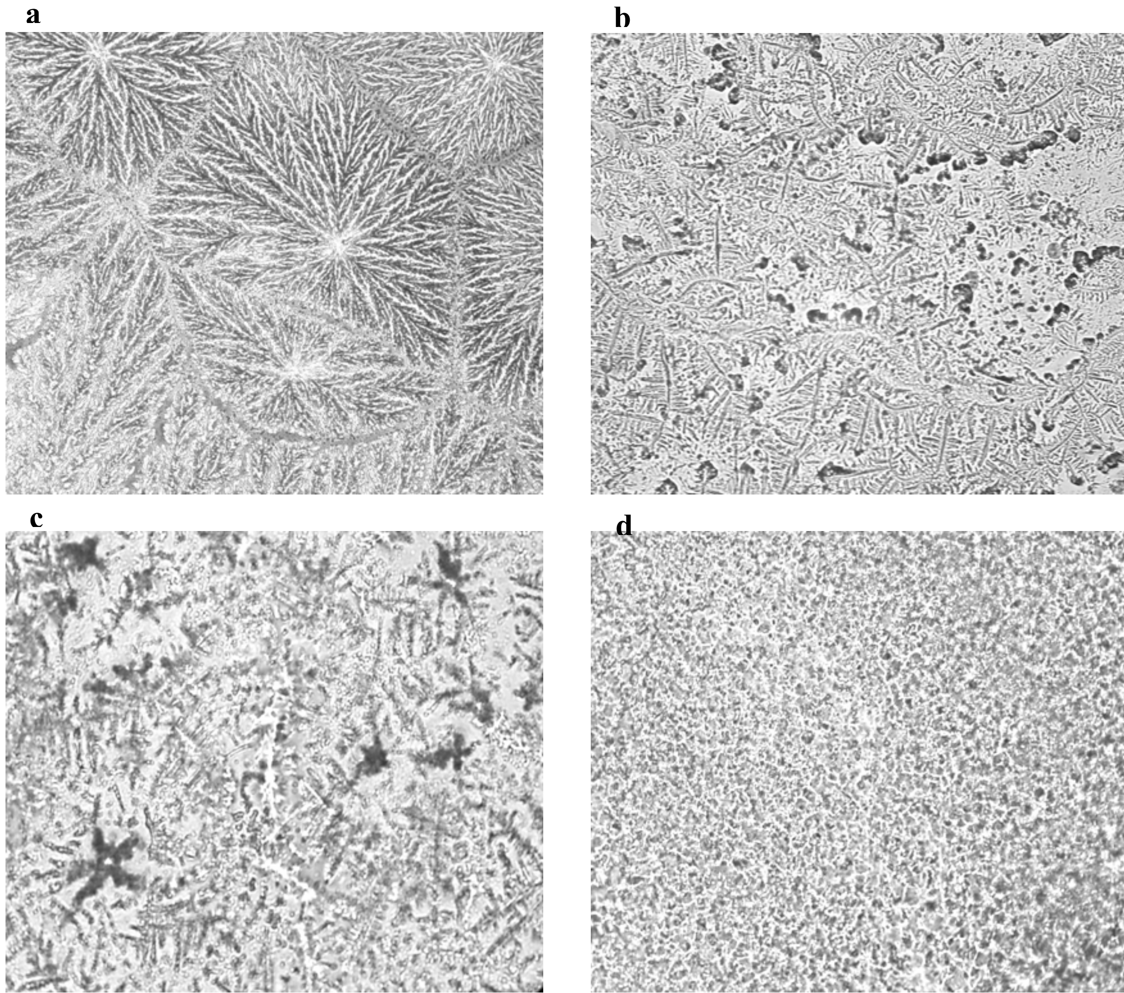

Figure 6-4: Tear ferning patterns in postmenopausal women

a) grade1 (uniform, abundant ferning, with no spaces between ferning); b) grade 2 (abundant ferning, with some empty spaces between ferning; c) grade 3 (partial ferning, with large empty spaces between ferning); d) grade 4 (absence of ferning)

When an attempt was made to correlate osmolality with ferning patterns, no significant correlation was found (DE: r=0.12; p>0.05, NDE: r=-0.17; p>0.05). There was also no significant correlation found between OSDI scores with osmolality (DE: r=0.40; p=0.06, NDE: r=-0.30; p=0.17) or ferning grades (Ferning: DE: r=0.25; p=0.35, NDE: r=-0.03; p=0.90).

6.5 Discussion

In this study, two groups of PMW were evaluated for tear osmolality using a new nanolitre osmometer that employs the freezing point depression technique. Tear ferning patterns of these individuals were also evaluated and classified based on the Rolando grading system.

Our results for tear osmolality are in accordance with studies previously undertaken. Reports have suggested that the average tear film osmolality values for non dry-eyed individuals assessed using the freezing point depression osmometry and vapor pressure osmolality is 302 ± 9.7 mOsm/Kg, with a range of 283 – 318 mOsm/Kg.[36] Large variations have been recorded in the osmolality values for dry-eyed individuals, with a mean value of 326 ± 22.1 mOsm/Kg and a range from 314 – 365 mOsm/Kg.[36] This same study, based on a meta analysis on published data in determining referent values for diagnosis of dry eye, has shown the cut off value to be 315.6 mOsm/Kg.[36] Our results showed a similar trend, with higher values for the dry-eyed participants (328.1 vs 315.1), with our mean for non dry-eyed osmolality being slightly higher than the mean reported by Tomlinson et al.[36] This could be due to the fact that the tear production, flow and volume reduce with older age,[37, 38] hence causing an increase in the solutes present in the tears and thereby increasing tear osmolality. In this study, all participants were older than 50 years of age and thus a slightly higher than normal osmolality would be expected. However, to-date normal referent values for varying age groups does not exist and this warrants further study.

Most of the asymptomatic candidates in this study did not only fall into the ferning grade I category, which re-emphasizes the fact that there is a tendency towards less ferning in people aged over 40 years of age, regardless of their symptoms.[29] There was no significant correlation between OSDI score and ferning grade, indicating that mild to moderate forms of dry eye show varying ferning grades. However, we were able to demonstrate a significant overall difference between the two groups, suggesting that "extremes" do show differences, with a general movement from grade I to grade IV as the symptoms increase. Tear ferning is a non-invasive laboratory technique that can be performed in a clinical as well as research set up. However, care must be taken to maintain the room temperature and humidity levels while drying the tear drop on the glass slide, as this process can be greatly affected by environmental factors.[27] This must be ensured, especially when diurnal variation studies to study ferning patterns are being undertaken.

A previous study[39] was not able to show any significant correlation between dryness symptoms and tear ferning patterns, whereas they were able to show a correlation between impression cytology and symptoms. Previous studies have shown that dryness and ferning patterns are related,[29, 40] which is supportive of our findings, which indicate that subjects with symptoms of dry eye show poorer ferning patterns.

Our data were unable to show a significant correlation between tear osmolality and ferning patterns. The spread of the data clearly shows that not all the participants who reported with higher osmolality values had poor ferning patterns. This is the first paper that we are aware of that has addressed this issue in human subjects, particularly PMW

and these data suggest that the factors that produce alterations in osmolality and ferning are independent of each other.

6.6 Conclusion

Dry eye is a common ocular surface disorder with diverse etiology.[41] A novel nanolitre osmometer, which requires <1 μl of tears, is a useful tool to determine tear film osmolality. Tremendous improvements and refinements have been made to measure osmolality in a very quick and reliable manner, which can be used as a simple clinical test for assessment of DES. Tear ferning is a rapid, simple, non-invasive laboratory procedure that indicates the altered tear quality in dry eye conditions and can provide valuable additional data in the diagnosis of subjects with DES. Further research is required to study the tear film osmolality and ferning patterns on severe dry eyed groups and the effects of diurnal variation, in addition to studies to determine appropriate referent osmolality values for differing age groups.

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7 Tear Lipocalin and Lysozyme Concentrations in Postmenopausal Women

Srinivasan S, Joyce E, Boone A, Simpson T, Jones L, Senchyna M. Tear lipocalin and lysozyme concentrations in postmenopausal women. In submission (Experimental Eye Research)

7.1 Abstract

Purpose: To investigate the potential relationship between subjective symptoms, tear volume, and tear break up time with tear film lipocalin and lysozyme concentrations in a group of symptomatic dry-eyed postmenopausal women (PMW), compared to asymptomatic controls.

Methods: Eighty five healthy PMW (>50 years of age) were categorized as mild or moderate dry eye (DE), or asymptomatic (no symptoms of dry eye (NDE) based on their responses to the OSDI© questionnaire. Non invasive tear breakup time (NITBUT) and tear secretion were measured. Tears were collected via capillary tube and an eye wash method. Tear lysozyme and lipocalin concentrations were determined via Western blotting.

Results: OSDI responses revealed 16 mild DE, 30 moderate DE, and 39 NDE. The OSDI total score as well as the sub scores for the DE groups were significantly greater than the NDE group (p<0.001). The mild and moderate DE groups exhibited significantly shorter

NITBUTs compared to NDE ($p<0.004$). Tear secretion using PRT test was found to be significantly lower in the moderate DE group compared to NDE ($p <0.001$). No difference in tear lysozyme or lipocalin concentration was found between DE and NDE groups, irrespective of tear collection method, although method of collection significantly influenced absolute concentrations ($p<0.008$). Significant correlations were not found between symptoms or signs of dry eye compared to either lipocalin or lysozyme concentration.

Conclusion: Our data clearly demonstrated a lower NITBUT and tear secretion in DE individuals compared to NDE. No difference was found in tear film lipocalin or lysozyme concentration between DE and NDE individuals, irrespective of tear collection method. Comparison of clinical data with lipocalin and lysozyme concentrations failed to reveal statistically significant correlations.

7.2 Introduction

Due to the efforts of the 2007 Report of the International Dry Eye Workshop (DEWS), 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*".[1] It is estimated that 5% to over 35% of the population at various ages are affected by dry eye.[2] Data from large epidemiological studies of dry eye (the Women's Health Study[3] and the Physicians Health Study[4]), clearly suggest that the prevalence of dry eye is greater in women than in men, and that approximately 3.2 million women and 1.6 million men aged 50 years or older suffer from moderate to severe dry eye. Identifying risk factors for dry eye is complicated, in part, due to the multi-etiological nature of dry eye. Evidence does suggest that hormonal status, and in particular sex steroids, play a role in ocular surface homeostasis and function. Not only is dry eye associated with menopause but also menopausal hormone therapy increases the prevalence of dry eye over menopausal women not receiving therapy.[1]

Diagnosis of dry eye is currently based on clinical findings, in particular tear-film break-up time, tear secretion or volume, ocular surface staining and patient symptoms.[5-7] However, several studies have reported very poor correlations between the classical signs and symptoms of dry eye,[8-12] in addition to poor test reproducibility.[13] This poor correlation may in part be due to the lack of understanding of symptoms and how they relate to test results.[13-15] However, other contributing factors may be the multi-etiological nature of dry eye, as well as a lack of standardization of diagnostic tests and symptom questionnaires.[6] Due to these issues, an enhanced biochemical understanding of the

pathophysiological / biochemical processes underlying dry eye would enable both the proper utilization and continued development of specific and sensitive diagnostic tests to facilitate objective reproducible and standardized diagnostic tests and methods to evaluate treatment efficacy.

The tear film, which is a critical component of the ocular surface function unit, is a dynamic entity. The production and turnover of tears complete in mucins, proteins, lipids, lipoproteins, glycolipids and aqueous is essential to providing the ocular surface with microbiological protection, nutrition, lubrication and cleansing as well as maintenance of visual acuity. Based on our current understanding, alterations in volume and/or composition of the tear film can lead to reduced stability and function and as a consequence manifest as dry eye disease.[16]

Alteration in expression of numerous proteins, lipids, mucins, inflammatory cytokines, matrix metalloproteases and growth factors have been implicated in dry eye.[16-24] However, there is little information characterizing changes in tear film composition and how such putative biochemical changes relate to tear function and/or signs and symptoms of dry eye. Of the data that does exist, reductions in tear film proteins such as lysozyme, lipocalin and lactoferrin have been described as markers of ocular surface disorders such as dry eye, conjunctivitis and blepharitis.[25-30] Lipocalin and lysozyme are particularly abundant in the complete tear film, where they assume numerous key functional and protective roles. Approximately one third of the protein content of the tears is made up of lipocalins,[31-35] which are members of the lipid binding protein super-family. Tear lipocalins bind and transport phospholipids, fatty acids, cholesterol, retinol and tocopherol, scavenge lipid products of inflammation and possess antimicrobial

activity.[31, 36-38] Lysozyme, which accounts for 20-40%[33, 39, 40] of total tear protein is best known for its anti-microbial activity.[35, 39, 41]

To date, no comprehensive study has been performed to assess tear film lysozyme or lipocalin concentration in postmenopausal women (PMW). Thus, the purpose of this study was to investigate the potential relationship between subjective symptoms, tear secretion and tear break up time with tear film lipocalin and lysozyme concentrations in a group of symptomatic dry-eyed PMW, compared to asymptomatic controls to gain insight into potential clinical – biochemical relationships and gauge the utility of these protein biomarkers for the diagnosis of dry eye.

7.3 Methods

7.3.1 Participants

Approval of this project was granted through the Office of Research Ethics at the University of Waterloo and all procedures adhered to the tenets of the Declaration of Helsinki. Participants were recruited at the Centre for Contact Lens Research, University of Waterloo, School of Optometry. Informed consent was obtained from all participants following explanation of purpose and procedures. A case history and complete ocular surface examination was performed to determine participant eligibility. Participants on hormone replacement therapy (HRT) were excluded, as were contact lens wearers and participants receiving any topical ocular medication or systemic medication known to exacerbate dry eye. Participants with history of blepharitis and/or active blepharitis were

also excluded from the study. For the purpose of this study, "postmenopausal" was defined as no menses for at least one year, not associated with hysterectomy.

7.3.2 Subjective symptoms

Eighty five healthy PMW (>50 years of age) were recruited. Participants completed the Allergan Ocular Surface Disease Index© (OSDI)[42] and were categorized as being symptomatic (46 individuals; age=63.2±9.5 yrs) or asymptomatic (39 individuals; age=59.9±7.3 yrs) of dry eye, based on their response to OSDI, as described in detail elsewhere[42] and in chapter 4.

7.3.3 Objective measurements

Tear stability was assessed by performing non invasive tear breakup time (NITBUT) using the ALCON Eyemap® model EH-290 topography system (ALCON, Inc., Forth Worth, Texas, USA) (explained in chapter 3 section 3.4).

Tear secretion was assessed using the Phenol Red Thread (PRT) test. The PRT (ZONE-QUICK, Showa Yakuhin Kako Co., Ltd. Tokyo, Japan) was left in the lower conjunctival fornix for 15 seconds. The length of colour change from yellow to red on the thread was measured in millimeters (explained in chapter 3 section 3.5).

Corneal and conjunctival staining could not be performed as a part of this study due to the nature of the study design, which included collection of tears and conjunctival impression cytology samples. The addition of fluorescein and/or other vital dyes would affect the subsequent analysis of various biomarkers and was thus not undertaken.

7.3.4 Analytical techniques

Reagents and Materials: All PhastSystem™ pre-cast gels, buffer strips, well combs, filter paper and ECL-Plus™ kits were purchased from GE Healthcare (Baie d'Urfe, QC, Canada). Immuno-Blot® PVDF (polyvinylidene difluoride) membrane and DC Protein Assay Kit® 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).

7.3.5 Tear collection

Tears were collected using a capillary tube from all subjects. In addition, to explore the utility of an alternative means of tear collection, 46 subjects (20 controls and 26 DE) also underwent a second collection by way of an eye wash. The two collections were separated by 15 – 25 minutes to allow for tear film regeneration.

Capillary Tear Collection: Using a graduated disposable 5 μl microcapillary tube (Wiretol-Micropipettes, Drummond Scientific Co., Broomall, PA, USA) up to 5 μl of tears / eye were collected from the inferior temporal tear meniscus of each participant as explained in chapter 3 section 3.6. A maximum of 5 min was allowed / eye for tear collection. Tears from both eyes were pooled and stored at -80°C until use. 0.5 μL of

tears diluted in 9.5 µL of distilled water and the DC Protein Assay Kit® (BioRad, Mississauga, ON, Canada) were used to determine total tear film protein. Duplicate samples were analyzed and data was read at 750 nm on a Multiskan Microplate spectrphotometer (Thermo Labsystems, Franklin, MA, USA) (explained in chapter 3 section 3.13.1).

Eye Wash Tear Collection: Tears were collected using a wash method as described elsewhere[24] and explained in chapter 3 section 3.10). Washes were pooled together and stored at -80°C until use. Total protein was determined as described above using 5 µL of eye wash.

Both tear sampling methods were conducted as carefully as possible to avoid reflex tearing. The time taken to collect the sample via capillary tube was less than three minutes, with most dry-eyed participants typically taking longer than the non dry-eyed subjects. Time taken for collection of eyewash samples were the same for both the groups. It approximately took one minute per eye to perform the eye wash collection.

7.3.6 Electrophoresis and immunoblotting

Quantification of tear lipocalin and lysozyme are explained in chapter 3 section 3.13.3 and appendix F.

7.4 Statistical analysis

Statistical analysis was performed using Microsoft Excel™ XLfit© software. All data are reported as mean ± standard deviation. Statistical differences between groups were identified by one-way ANOVA, and when necessary, Dunnett's comparison of

means and by Tukey's test. Significance was identified at $p<0.05$ ($\alpha = 0.05$). Pearson correlations between subjective and objective clinical measurements and concentration of lipocalin or lysozyme were calculated in Excel™.

7.5 Results

7.5.1 Subjective symptoms

In this study, the presence and severity of dry eye was determined based on symptoms only, as quantified by total OSDI score, using the following criteria: control (non-dry eye, NDE) OSDI score = 0-12; mild dry eye OSDI score = 13-22 and moderate dry eye OSDI score= 23-32 (Table 7-1). Based on this criterion, 39 subjects were defined as controls. Sixteen subjects presented with mild dry eye and the remainder (n=30) were classified as moderate. Mean ages (mean ± SD) of the control, mild and moderate groups were 59.5 ± 6.5, 63.1 ± 10.0 and 64.2 ± 8.9 years respectively. There was no significant difference between the ages of the three groups ($p=0.07$). The total OSDI scores for the two dry eyed groups were significantly higher compared to the NDE group ($p<0.0001$), as was the total score of the moderate group compared to the mild group ($p=0.03$). Analysis of the individual OSDI sub scores revealed a significantly elevated score for the mild and moderate DE groups in each category compared to the NDE group, with the exception of the Vision Related Function score, where there was no distinction between NDE and mild dry eye. Mild and moderate dry eye sub scores were statistically similar, with the exception of the Vision Related Function score, which was significantly elevated in the moderate group ($p<0.01$).

Table 7-1: Summary of Ocular Surface Disease Index© scores

OSDI scores	Overall			Subcategory		
	NDE (n = 39)	Mild Dry Eye (n = 16)	Moderate Dry Eye (n = 30)	p (α =0.05) (NDE vs Mild)	p (α =0.05) (NDE vs Moderate)	p (α =0.05) (Mild vs Moderate)
Total OSDI Score	7.4±7.7	18.9±9.2	27.2±14.4	**<0.001**	**<0.001**	**0.03**
Ocular Symptoms	7.6±8.4	18.1±13.5	25.3±19.5	**<0.006**	**<0.001**	0.17
Vision Related Functions	7.1±12.2	8.6±7.1	20.8±17.7	0.6	**<0.001**	**0.005**
Environmental Triggers	8.2±12.3	31.0±20.6	36.9±28.7	**<0.001**	**<0.001**	0.45

7.5.2 Objective measurements

Figure 7-1 reports the tear secretion values determined by the PRT test. Tear secretion in moderate DE subjects was significantly reduced compared to NDE subjects (p<0.0001). Tear secretion in mild DE subjects was slightly lower when compared to NDE, however statistical significance was not achieved (p=0.15). Figure 7-2 reports the NITBUT determined by the corneal topographer. NITBUT was significantly reduced in both mild (p=0.04) and moderate (p<0.001) DE subjects compared to the NDE group, and no difference was found between the two dry-eyed groups (p=0.73).

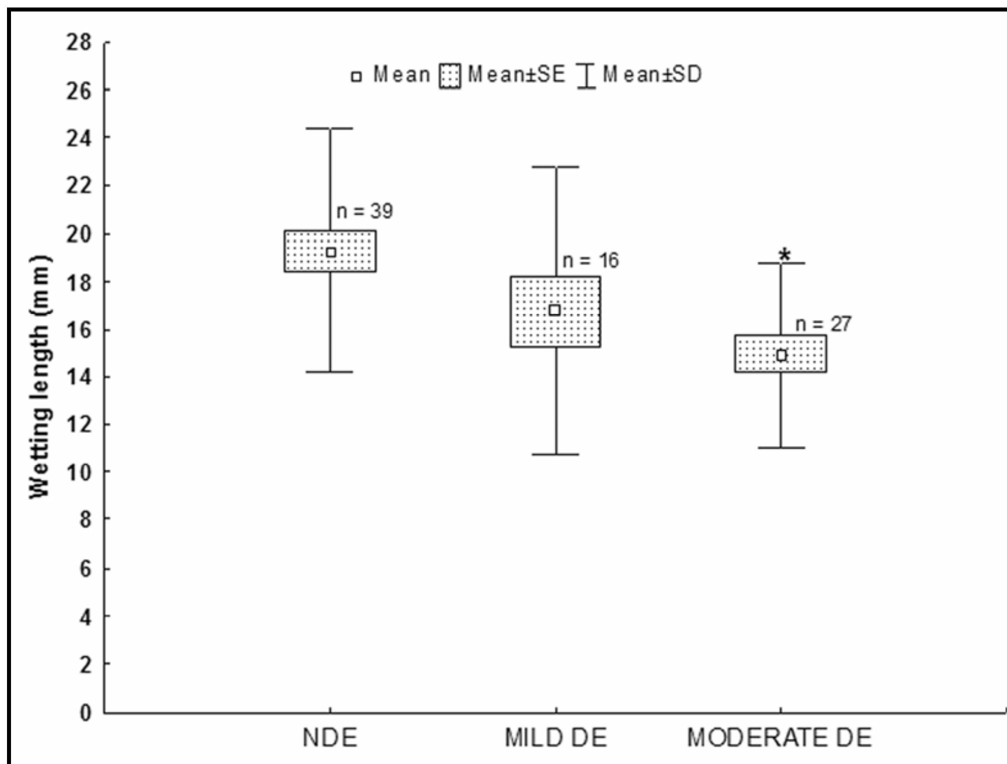

Figure 7-1: Box plots of tear secretion as measured by phenol red thread test

Inner boxes represent mean, dotted boxes represent standard error and whiskers represent standard deviation of phenol red thread test measurements from non dry-eyed subjects (NDE), mild dry eye and moderate dry eye subjects. * represent statistically significant difference relative to NDE.

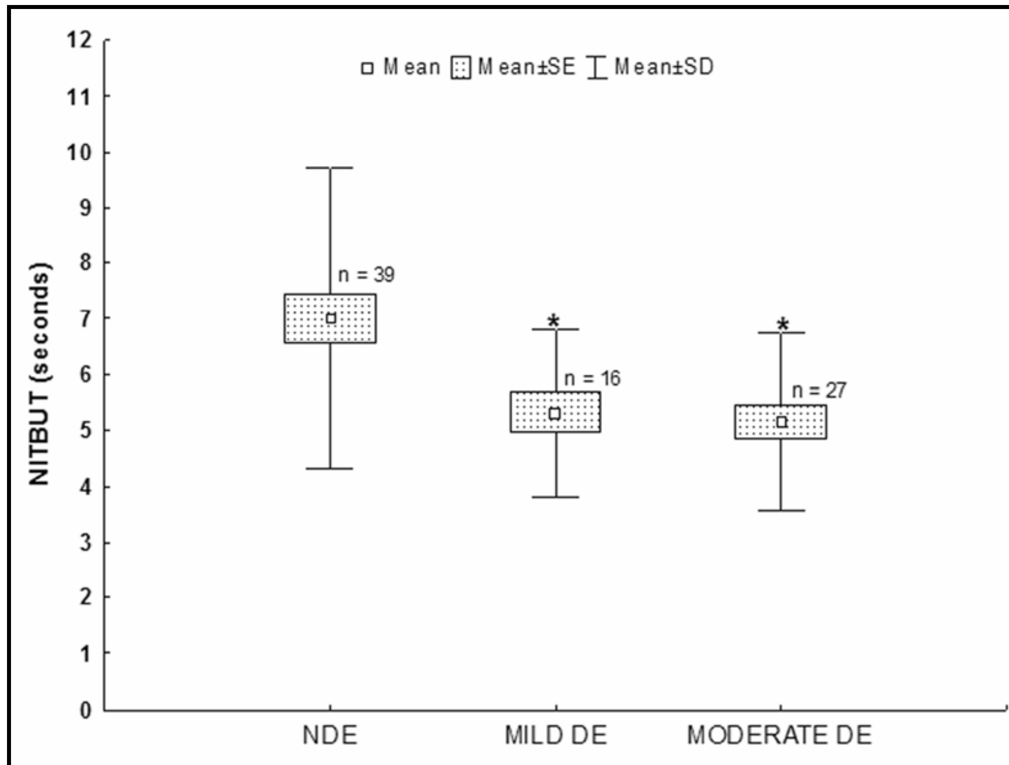

Figure 7-2: **Box plots of non invasive tear film break-up measurements**

Inner boxes represent mean, dotted boxes represent standard error and whiskers represent standard deviation of NITBUT measurements from non dry-eyed (NDE), mild dry eye and moderate dry eye subjects. * represent statistically significant difference relative to NDE.

7.5.3 Tear total protein

Figure 7-3 reports the total tear protein data in the tears collected by glass capillary tube. No difference in protein concentration was found comparing mild ($p=0.057$) or moderate ($p=0.895$) dry eyed tears to NDE tears, nor was a difference found between the two DE groups ($p=0.056$). Although protein concentration was lower in tears collected using the eye wash method due to dilution with saline, no difference ($p=0.26$) was found in protein concentration between the NDE (1.91 ± 0.94 µg/µL) and pooled DE groups (1.61 ± 0.91 µg/µL). Stratification of total protein values based on dry eye

symptom severity was not performed with eye wash data due to relatively small sample size of both DE groups.

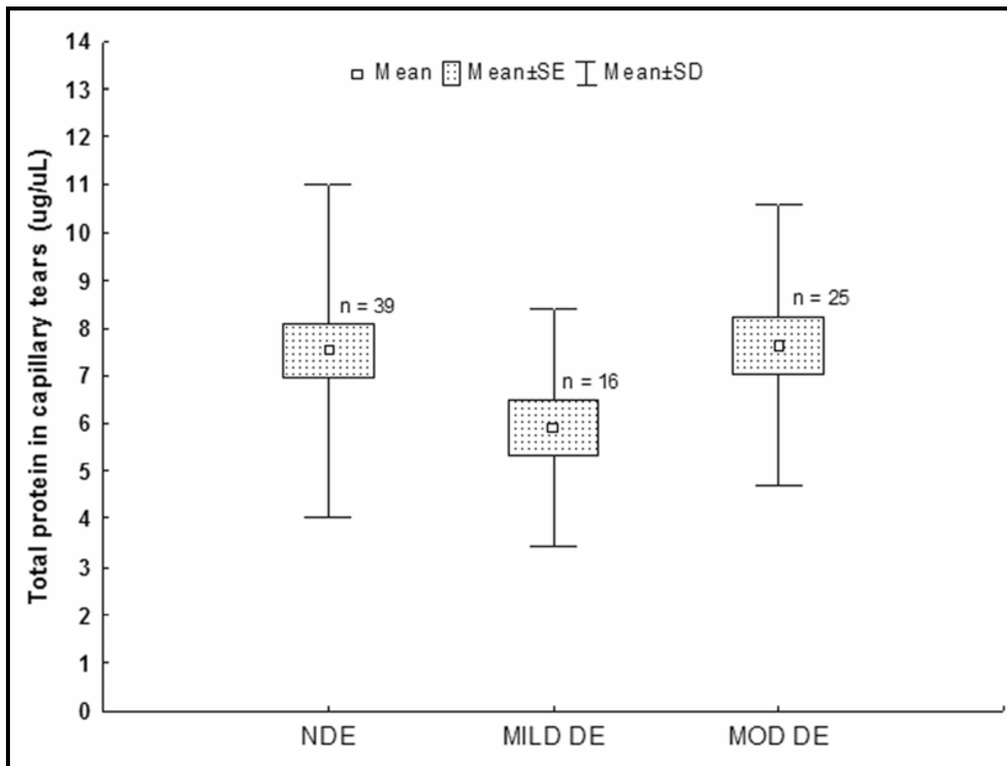

Figure 7-3: Box plots of tear film protein concentration

Inner boxes represent mean, dotted boxes represent standard error and whiskers represent standard deviation of tear film protein concentration from non dry-eyed (NDE), mild dry eye and moderate dry eye subjects. Tears were collected using a glass capillary tube.

7.5.4 Tear film lipocalin concentration

Figure 7-4 reports the tear film lipocalin concentration collected via glass capillary tube (n=74) and eyewash methods (n=46). No difference in lipocalin concentration was found between DE and NDE groups using tears collected via capillary tube (p=0.26) or eye wash (p=0.42). Paired comparison of the two tear collection techniques (n=43) revealed a significant difference in calculated tear lipocalin concentration (p=0.0004), where mean capillary concentration was 0.22 ± 0.12 AU and mean eye wash concentration was 0.15 ± 0.06 AU. Lipocalin concentration in relation to

symptom severity of dry eye was not examined with eye wash tear samples due to relatively small sample size of the two DE groups. A trend towards reduction in lipocalin concentration as symptom severity of dry eye increased was noted with tear samples collected with capillary tubes, as shown in Figure 7-5. No significant correlations were found between tear film lipocalin concentration and tear secretion, tear break up time or OSDI questionnaire response data (Table 7-2).

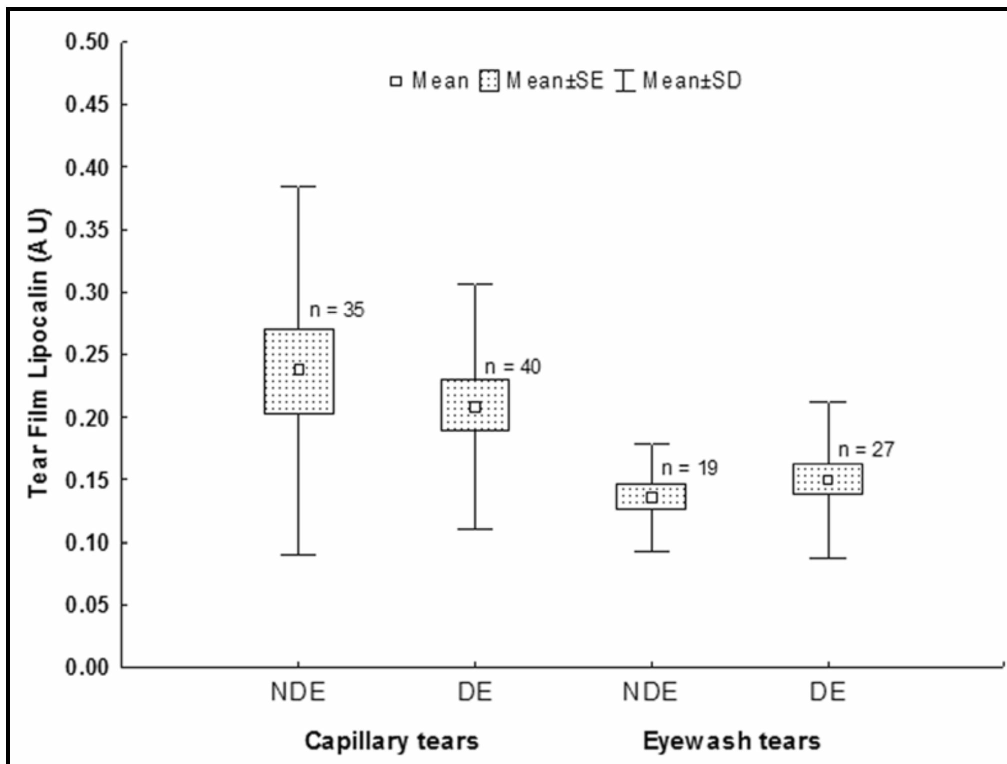

Figure 7-4: Box plots of mean tear film lipocalin concentration

Inner boxes represent mean, dotted boxes represent standard error and whiskers represent standard deviation of lipocalin measurements from non dry-eyed (NDE) dry-eyed (DE) subjects (participants with mild & moderate symptoms combined). Data from tears collected via capillary and eye wash methods are displayed for comparison.

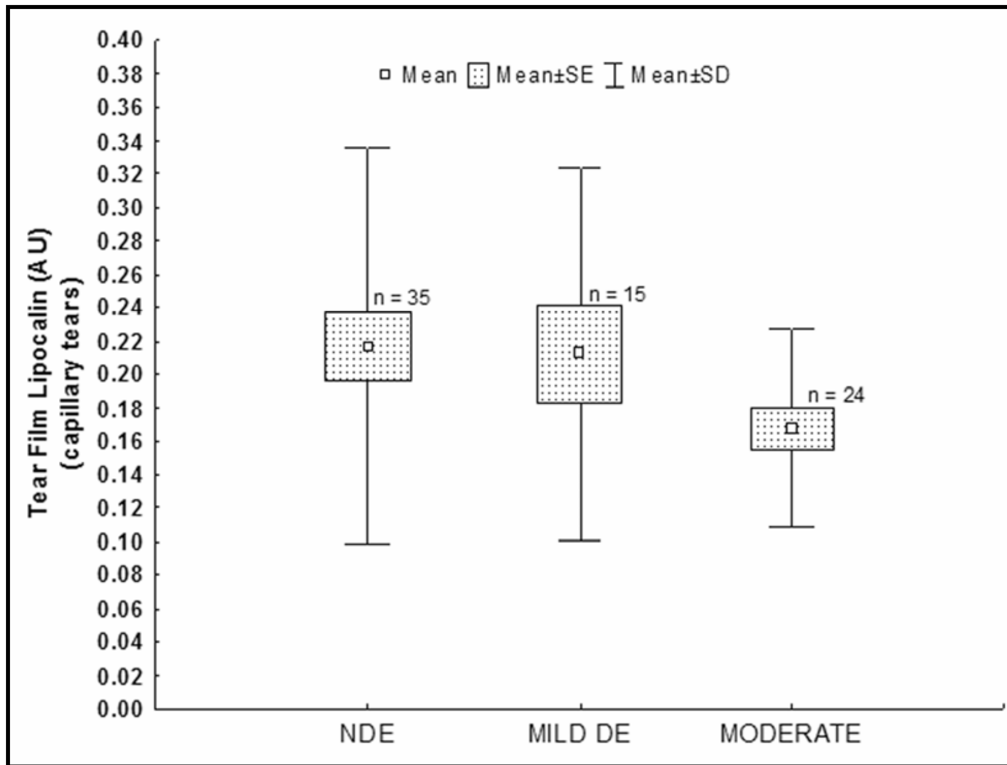

Figure 7-5: **Box plots of mean tear film lipocalin concentration as a function of dry eye symptom severity**

Inner boxes represent mean, dotted boxes represent standard error and whiskers represent standard deviation of lipocalin measurements from non dry-eyed (NDE), mild dry-eyed and moderate dry-eyed subjects. Tears were collected with capillary tubes.

7.5.5 Tear film lysozyme concentration

Figure 7-6 reports the tear film lysozyme concentration collected via glass capillary tube (n=43) and eyewash methods (n=38). No difference in lysozyme concentration was found between DE and NDE groups using tears collected via capillary tube (p=0.20) or eye wash (p=0.59). Lysozyme concentration in relation to symptom severity of dry eye was not examined due to relatively small sample size for both methods of tear collection. Paired comparison of the two tear collection techniques (n=37) revealed a significant difference in calculated tear lysozyme concentration (p=0.008), where mean capillary concentration was 0.30 ± 0.09 µg/µg total tear protein

and mean eye wash concentration was 0.25 ± 0.08 µg/µg total tear protein. No significant correlations were found between tear film lysozyme concentration and tear secretion, tear break up time or OSDI questionnaire response data (Table 7-2). Correlation analysis between OSDI total scores for the three groups (normal, mildly symptomatic and moderately symptomatic dry eye) and tear secretion, tear break up time or lipocalin via capillary tube showed weak and insignificant correlation. Similar results were found for the correlation analysis between OSDI sub scores and tear function test.

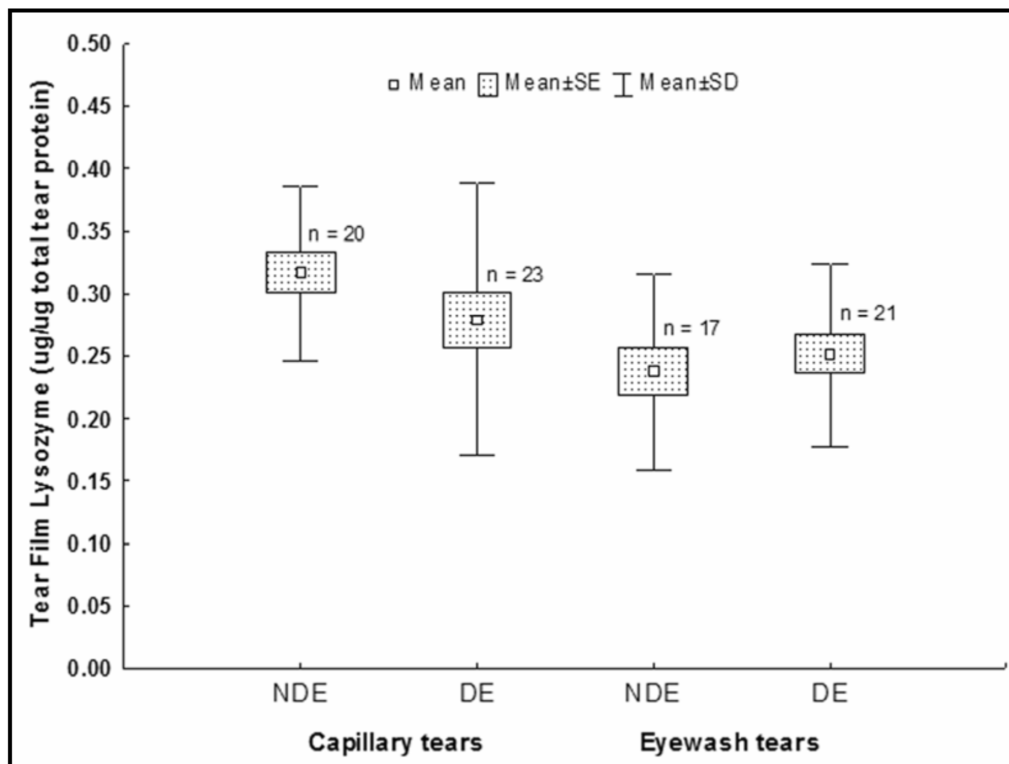

Figure 7-6: Box plots of mean tear film lysozyme concentration

Inner boxes represent mean, dotted boxes represent standard error and whiskers represent standard deviation of lysozyme measurements from non dry-eyed (NDE) and dry-eyed (DE) subjects (participants with mild & moderate symptoms combined). Data from tears collected via capillary and eye wash methods are displayed for comparison.

Table 7-2: Summary of correlations between subjective and objective measures of dry eye and tear film lipocalin and lysozyme concentrations

Clinical Measurements	Lipocalin Concentration in Tears Collected with Capillary Tube (n=75)	Lipocalin Concentration in Tears Collected via Eye Wash (n=42)	Lysozyme Concentration in Tears Collected with Capillary Tube (n=43)	Lysozyme Concentration in Tears Collected via Eye Wash (n=38)
PRT Test	0.181	0.032	-0.094	-0.184
NITBUT	0.072	0.002	0.241	0.058
Total OSDI Score	-0.174	0.175	-0.347	0.150

7.6 Discussion

Several studies have suggested that various tear film proteins may serve as surrogate endpoints for evaluation of ocular surface disorders, and the quantitation of tear lysozyme has recently been proposed as one of many potential clinical tests used in the assessment of dry eye.[5] In this study, we quantified the concentration of tear film lysozyme and lipocalin in two groups of PMW, with and without symptoms of dry eye, to gain insight into the diagnostic sensitivity and selectivity of these biomarkers and to investigate potential relationships between protein concentrations and clinical signs and symptoms of dry eye.

We chose to stratify subjects solely on symptoms as quantified by the OSDI questionnaire. It is a validated questionnaire recommended by the DEWS 2007 report and it is one way of potentially classifying participants based on the symptom scores. Based on the OSDI criteria, we enrolled three statistically distinct groups: those without symptoms of dry eye and those with either mild or moderate dry eye. Review of objective measurements suggested that both tear secretion and NITBUT were significantly reduced in moderately symptomatic dry-eyed subjects relative to controls. Additionally, mildly symptomatic dry-eyed subjects presented significantly reduced NITBUT relative to controls. An evaluation of our PRT data does indicate that the values reported are higher than those anticipated, with the values for DE group being higher than the cut-off that have been proposed by previous authors.[13, 43, 44], The suggestion of a cut-off value comes from a study that included a population with a wide age range and both genders[43, 44], which is quite different to this study, which includes only older female subjects. As one would expect, age typically results in reduced tear volume.[45, 46]

However, a recent study has shown that older individuals tend to exhibit a higher tear meniscus height (and hence tear volume), due to age-related constriction of the puncta, resulting in limited drainage of tears from the ocular surface.[47] Thus, it is entirely feasible that our PRT data for our older group of PMW is appropriate, potentially due, in part, to punctal stenosis. However, this requires further investigation. Taken together, it appears that alterations in tear stability and secretion are involved in the pathophysiology of dry eye within the PMW population. Further research is currently underway to explore correlations within this data set.

With respect to protein biomarkers, Dougherty and McCulley[48] reported that blepharitis patients with clinically diagnosed KCS had a lower mean tear film lysozyme concentration compared to either blepharitis patients with no KCS or control subjects. In chronic smokers, Satici et al[49] reported that tear film break time, mean Schirmer I scores and tear lysozyme concentration were statistically lower compared to a group of non-smokers and thus concluded that each measurement was an assessment of ocular surface damage. Whether a dilution effect was present due to excessive tearing associated with smoking was not ruled out. deLuise and Tabbara[27] described a trend favoring reduced tear lysozyme concentration in dry eyed individuals compared to a control population although statistical significance was not achieved due to considerable data overlap between the two groups. Markusse et al[50] also found that despite a trend of reduced lysozyme concentration in primary Sjögren' subjects compared to controls, data overlap suggested that lysozyme quantitation lacked the required specificity for diagnostic use. Data reported in this study are in agreement with these latter two studies, as our results suggest that no difference in tear lysozyme concentration exists in dry-eyed PMW

compared to asymptomatic controls, irrespective of method of tear collection. Few studies have shown a difference in the lysozyme levels in tears.[21, 51] These differences in results could be due to the population studied, age, tear collection method, classification system for what constitutes “dry eye”, or the analytical method used.

Tear lipocalin has been proposed as a marker of lacrimal function,[52, 53] as well as playing a key role in maintaining tear stability.[29, 53, 54] In a study of intolerant contact lens wearers,[29] it was found that tear lipocalin concentration was significantly elevated compared to a control group of tolerant wearers. The authors suggested that the increase in lipocalin may be in response to lipid by-products circulating in the tear film. That tear film instability may result from the altered tear biochemistry was supported by the finding that lipocalin concentration was significantly correlated with NITBUT (r=0.440; 0.036). Evans et al[53] reported that tear film lipocalin concentration in a group of PMW not using HRT was not significantly different to a post menopausal control group, as well as a group of hysterectomized women receiving HRT and a group of age-matched men. The authors also reported that the range of lipocalin concentrations was greatest in the post menopausal group not using HRT and suggest that HRT may smooth out fluctuating hormone levels associated with menopause and that estrogen may have an ability to regulate the secretion of lipocalin from the lacrimal gland. Our data are consistent with the work of Evans et al,[53] in that we found no difference in tear lipocalin concentration in postmenopausal dry-eyed women compared to asymptomatic controls, irrespective of method of tear collection.

We compared both lysozyme and lipocalin data with clinical results and found no correlation with OSDI response data, NITBUT or tear secretion. Although our work demonstrates that PMW with symptoms of dry eye had reduced tear stability and capacity to secrete tears, tears that were produced were biochemically normal with respect to two major lacrimal gland proteins (lysozyme and lipocalin), as well as total protein content.

As a secondary objective we sought to investigate the utility of two different tear collection methods. Although the use of glass capillary tubes is perhaps the most common technique, an eye wash method provides a user friendly, safe, rapid and - in the case of severe aqueous deficient dry eye - more realistic means of collecting tears. We found that although both tear collection methods provided the same conclusion of no difference in tear lysozyme or lipocalin concentration between study groups, the absolute concentrations calculated were statistically different, with higher values associated with capillary collection in both cases. Studies comparing capillary collection to Schirmer I or polyester rod collection have found that tear protein concentrations are greater with the latter techniques, potentially due to contributions from intercellular constituents and/or absorption characteristics.[55-57] We can speculate that an eye wash technique may add a minimal dilution variable to overall protein yield, thus resulting in slightly lower absolute values when data is normalized to total protein, as was done in this study. Our work, as well as that reported in the literature, highlights the importance of specifying collection technique in the comparison of data sets. Furthermore, variables such as the biomarker under study, the subject population and post-collection analytical methods may all influence or be affected by a given tear collection technique. Thus, prior to adopting a method of tear collection, all such variables should be considered.

7.7 Conclusion

The quantitation of tear biomarkers offers potential advantages of specificity and reproducibility that could provide valuable information to supplement conventional dry eye diagnostic criteria. In this study, we investigated the use of lipocalin and lysozyme for such a purpose. We can conclude from these data that within a PMW population both proteins are invariant, irrespective of the presence and severity of dry eye symptoms. In addition, the concentration of either protein was not associated with tear stability or secretion. That previous studies have shown a difference in tear protein concentration and /or correlation with tear dynamics may be attributed to the subgroup of dry eye subjects studied. This is the first comprehensive study of lysozyme and lipocalin in dry-eyed PMW and our results suggest that neither protein would offer utility as a biomarker of dry eye.

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8 Method Optimization for the Isolation of Total RNA and Total Protein from Human Conjunctival Epithelial Cells Collected via Impression Cytology

Srinivasan S, Senchyna M, Heikkila E, Jones LW. Method optimization for the isolation of total RNA and total protein from human conjunctival epithelial cells collected via impression cytology. In submission (Molecular Vision)

8.1 Abstract

Purpose: To optimize a technique for the isolation of total RNA and total protein derived from human conjunctival epithelial cells collected *in situ* via impression cytology.

Methods: Conjunctival epithelial cells were collected via impression cytology (CIC) using either Millipore (MP) or Poly Ether Sulfone (PES) membranes. RNA Isolation: Following collection with either MP or PES, total RNA was isolated using one of two commercially available methods: TRIzol™ (TZ) (Life Technologies) or RNeasy™ Mini (RN) (Qiagen). RNA concentration and integrity was assessed. RT-PCR of mRNAs coding for MUC1 and the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was performed to confirm the collection of high quality, DNA-free RNA. Protein Isolation: Following CIC with (MP), samples were either frozen immediately or soaked in extraction buffer and then frozen. Total protein was isolated following the

addition of extraction buffer and boiling. The supernatant was collected and frozen for future use. Total protein was quantified with BioRad's DC™ Protein Assay. Western blotting of a constitutively expressed protein (lipoxygenase type 2 protein (LOX2)) was performed to confirm the collection of intact total protein.

Results: When the TZ and RN methods were compared, total RNA yield was greater with the MP membrane ($p=0.05$). However, both membranes consistently provided high quality ($\lambda 260:280 > 1.8$) RNA, with no significant difference between the kits (p=NS). All RNA isolated with the TZ and RN methods demonstrated positive amplification of MUC1 and GAPDH mRNAs as assessed by RT-PCR. The average yield of protein from a single membrane ranged from 3 μg to 64 μg. Positive identification of LOX2 protein via Western blotting confirmed the collection of intact total protein.

Conclusion: For RNA isolation: MP membranes processed with either the TZ or RN methods are equally efficient for the isolation of high quality RNA from conjunctival cells collected *in situ*. The RN method is recommended, due to enhanced speed as well as on-column isolation and DNase digestion capabilities. For total protein isolation, the use of CIC using MP membranes followed by immediate freezing and then extraction and processing with methods optimized by our laboratory facilitates the collection of total protein from human conjunctival cells. Both methods will prove very useful to assess the expression of a variety of proteins involved in both normal and pathophysiological functions of the human ocular surface.

8.2 Introduction

Conjunctival Impression Cytology (CIC) is a relatively simple, practical and minimally invasive technique, allowing the collection of 1 to 3 layers of cells from the conjunctival surface.[1-6] This technique is rapid, convenient and is widely performed on subjects to confirm a variety of ocular surface diseases and to monitor changes in the conjunctival surface over a period of time.[3, 7] CIC has been used since the 1970's[6] as a diagnostic procedure for the detection and grading of squamous neoplasia [8-10], dry eye syndrome[6, 11] and squamous metaplasia[4, 6, 12, 13] and remains used as a diagnostic procedure to this day.

Significant biochemical insight into human ocular surface physiology and pathophysiology can be gained through the analysis of genes and proteins expressed in conjunctival epithelial cells that are collected via impression cytology. Researchers have used a variety of different membranes to collect the samples, including cellulose acetate membranes of different pore sizes and Biopore membrane devices.[12, 14, 15] Among all these membranes, Millipore membrane (MP) (Millipore™, Billerica, Massachusetts, USA) and Poly Ether Sulphone membranes[16-19] (PES) (Gelman Laboratory, Supor® 200, Ann Arbor, MI, USA) have been most commonly used.

Isolation of intact RNA in sufficient quantity is essential for accurate gene expression analysis. However, RNA quality and yield can vary significantly depending on the cell source and method of RNA isolation. Even though elaborate procedures have been developed for RNA isolation, working with RNA is a practical challenge. RNA is well known to be highly susceptible to degradation due to its chemical structure and also because of contaminating RNAse from the environment.[20] Hence it is necessary to use a

reliable, quick and user friendly method of RNA isolation to obtain high quality RNA from CIC samples. One method that has been used frequently to isolate RNA from conjunctival or corneal cells is the TRIzol™ (TZ) (Life Technologies) RNA isolation technique.[19, 21-27] The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi.[28] Another fast and simple method for the preparation of up to 100µg total RNA from animal cells and tissues is the RNeasy™ Mini (RN) (Qiagen). This kit is designed to isolate total RNA from small quantities of starting material.[16-18, 20, 29] This isolation technique combines the selective binding properties of a silica gel based membrane with the speed of microspin technology.

Similarly, extraction and characterization of protein expression from epithelial cell populations is also dependant on the appropriate method used for isolation. To date, there is no simple method reported for extracting total protein from CIC samples.

The purpose of this study was to optimize techniques for the isolation of both high quality total RNA and total protein derived from human conjunctival epithelial cells collected via CIC. As objective measures to assess yield and quality the following were required:

a) RNA integrity gel to confirm the presence of intact RNA.
b) RT-PCR of two mRNAs known to be expressed in the conjunctiva (GAPDH and MUC1) to confirm intact RNA and the lack of DNA contamination).
c) Western blotting for the constitutively expressed 15-Lipoxygenase 2 (LOX 2) protein to confirm the collection of intact protein.

8.3 Methods

8.3.1 Isolation of RNA

Conjunctival epithelial cells were collected from both eyes of 12 human volunteers (different from the postmenopausal women studies) (6 per kit) via CIC using either Millipore (MP) or Poly Ether Sulfone (PES) membranes. (Pore size: MP = 0.45 µm, PES = 0.2 µm). After instillation of topical anesthesia, 10mm diameter filter paper discs were applied to the superior and temporal bulbar conjunctiva of each eye for five seconds (chapter 3 section 3.11.2). Following cell collection, filter papers were immediately immersed in extraction buffer consistent with the method being evaluated. The filters were vortexed vigorously for 10 seconds and then immediately placed on dry ice until placed in a -80ºC freezer for long term storage until further processing. The methods evaluated for total RNA isolation were: TRIzol™ (TZ) (Life Technologies) and RNeasy™ Mini (RN) (Qiagen). 100 µL of TRIzol reagent was used per isolation for TZ whereas 1.0 mL of RLT lysis buffer containing 1% β- mercaptoethanol was used for RN.[16, 17] RNA extraction was carried out following manufacturers recommendations for both kits with few modifications. The step wise procedure of RNA isolation for RNeasy™ Mini (RN) (Qiagen) is explained in appendix G. The procedure for RNA isolation using TRIzol™ (TZ) (Life Technologies) was undertaken using the following steps: CIC samples were soaked in 100µl of TRIzol and frozen in -80°C until further isolation. Samples were not stored for more than 30 days based on manufacturer's recommendations. Samples were allowed to come to room temperature and vortexed for 15 seconds. The reagent was passed through several times (at least 5 times) using a pipette that was set for at 90µl and was left in room temperature for 5 minutes. 20µl of

chloroform was added and vortexed for 15 seconds. Following 15 seconds of vortexing, the sample was allowed to sit at room temperature for 3 minutes. Samples were spun at 11,000g for 15 mins at 4º C, the upper colorless phase was transferred into a new sterile eppendorf and 10µg of glycogen was added. 50µl of isopropanol was then added and the sample was vortexed for 10 seconds followed by a 10 minute incubation at room temperature and centrifugations at 11,000g for 10 minutes at 4ºC. Supernatant was removed and 500µl of 75% ethanol was added. Then the sample was vortexed for 5 seconds and spun at 7,500g for 5 min at 4ºC. Ethanol was removed and the sample was desiccated to dryness. RNA was dissolved in 40µl of water and vortexed gently for 3-5 seconds, following which a quick spin for 3-5 seconds was given. Samples were then heated at 55º C for 10 minutes (the time and the temperature were strictly adhered), vortexed gently for 3-5 seconds and spun for 3-5 seconds. 5 µl of the sample was used for spectrometry (chapter 3 section 3.13.7) and the remaining sample was stored in -80°C for RT-PCR.

The two filter papers from the same eye were processed together in the same isolation. Both eyes from the same volunteer were processed in parallel (Figure 8-1). Samples isolated using the RN kit was subjected to on-column DNase-digestion using the supplied RNAse-free DNAse. Samples isolated with TZ were subjected to the addition of 10 µg glycogen (Life Technologies) and RNase-free DNase-digestion (Life Technologies) as suggested by the manufacturer. The concentration and integrity (λ260:280) of RNA was assessed via UV spectrophotometry. RNA integrity was additionally assessed via electrophoresis on 1% agarose-formaldehyde denaturing gels, followed by ethidium bromide staining.

RT-PCR of mRNAs coding for MUC1 and the housekeeping gene GAPDH was performed to confirm the collection of high quality, DNA-free RNA.

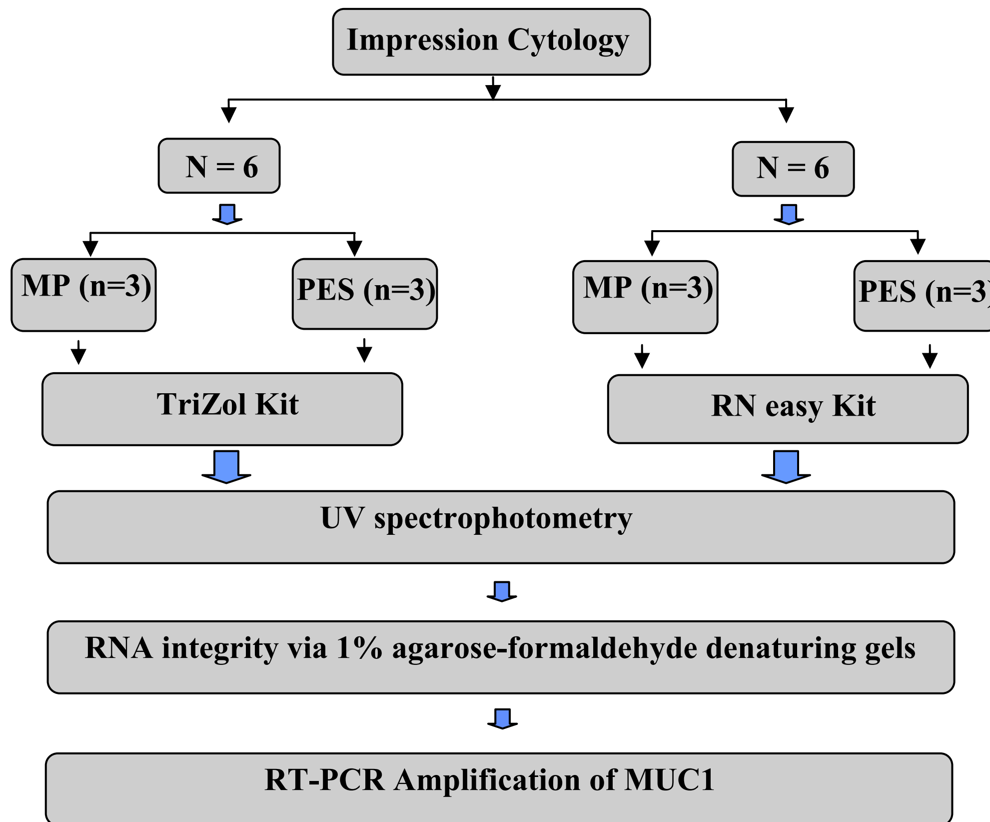

Figure 8-1: Flowchart showing the schematic of sample processing from the CIC disc for method optimization of RNA isolation

8.3.2 Isolation of total protein

Conjunctival epithelial cells were collected from both eyes of 12 human volunteers (different from the postmenopausal women studies) via CIC using 10mm Millipore (MP) membranes. Superior and temporal samples were always kept separate. Samples from each eye were processed separately. One set of CIC samples collected

from 6 volunteers were snap frozen in dry ice until further processing, while the discs collected from the remaining 6 volunteers were processed immediately as described below and then frozen at -70º C (Figure 8-2). CIC samples were laid on a glass plate with adhered cells facing up. Ten µL of Extraction Buffer (EB), containing 50mM Tris and 1% SDS, or EB + 1X Complete™ Protease Inhibitor Cocktail (PI) (Roche Diagnostics, 1X concentration) was applied to the cells.

The discs were cut up using a scalpel into 1mm pieces, added to an eppendorf tube containing an additional 40µL of EB or EB + PI, boiled for 10 minutes, spun at 14000g , aliquoted , and stored at -70º C. Total protein from 10µL of the sample was quantified using the BioRad's DC™ Protein Assay (appendix D). Absorbances were read at 750 nm on a Multiskan Microplate Spectrphotometer (Thermo Labsystems, Franklin, MA, USA. Western blotting of LOX 2 was performed to confirm the collection of intact protein. To facilitate quantitation of tear samples, standard curves were run on each gel. A titration (500 to 31.25 pg/µL) of LOX 2 standards (Recombinant 15-LOX Form-2 standard, Oxford Biomedical Research) was used. LOX 2 standards was used to quantify the amount of LOX 2 per sample. IC samples were diluted to 1µg/µL, 0.5µg/µL and 0.25µg/µL. 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).

LOX 2 was identified using LX25 primary antibody (Oxford Biomedical Research) (1:10,000) in TBS + 0.05% Tween 20 (TBS-T) for 4 hours at room temperature, followed by goat anti rabbit IgG secondary antibody (1:20 000) (Jackson Immuno Research) in TBS-T with 1% blotto (skimmed milk powder + TBS-T) for 2

hour. Immunoreactivity was visualized by incubating with ECL Plus® chemiluminescent substrate (Amersham-Pharmacia Biotech). Optical densities of the resulting bands were quantified from digitized images created with a Molecular® Dynamics Storm™ 840 Imager using ImageQuant™ 5.1.

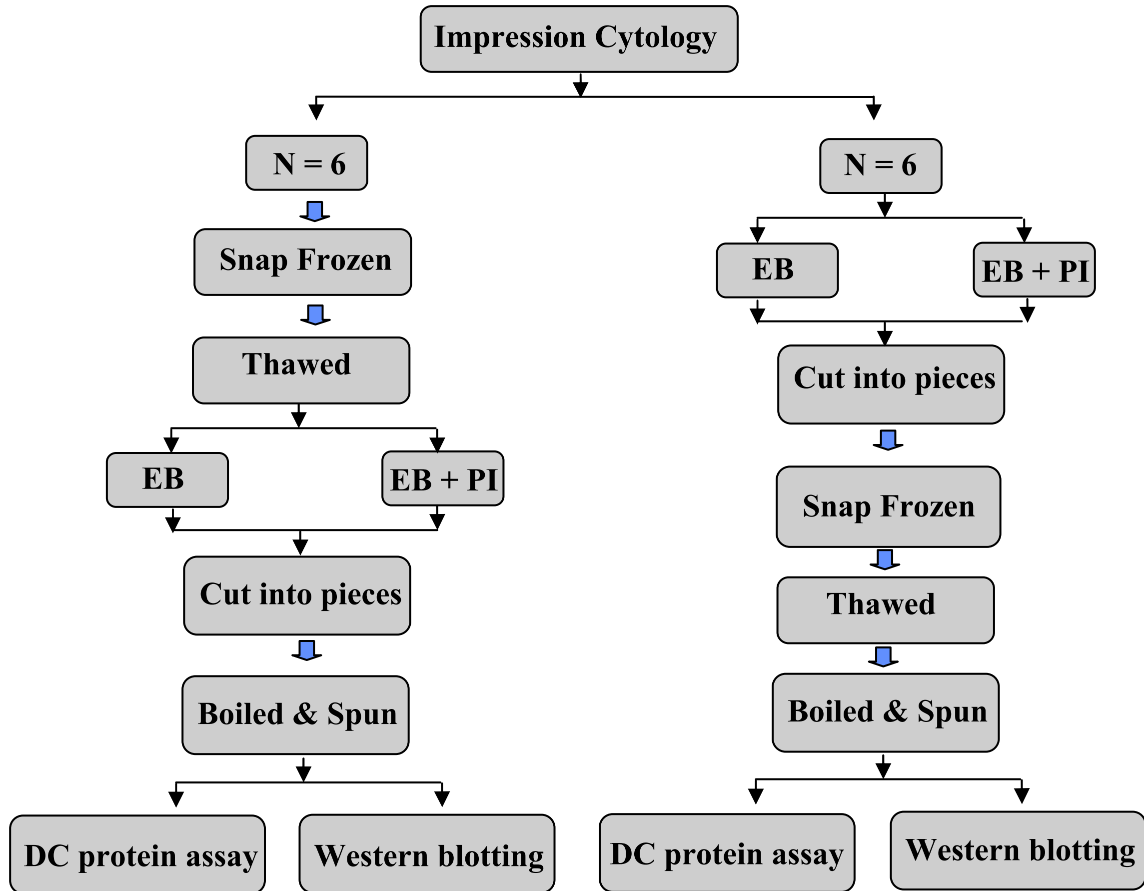

Figure 8-2: Flowchart showing the schematic of sample processing from the CIC disc for method optimization of protein

8.4 Statistical analysis

One-way analysis of variance was performed on all data to assess differences between the RNA isolation kits and the filter papers and protein isolation methods (significance taken at a value of $p<0.05$).

8.5 Results

Table 8-1 summarises the RNA quality and yields obtained using the TZ and RN methods combined with MP and PES membranes. As indicated in Table 8-1, total RNA yield was greater with the MP membrane ($p=0.05$), however, both membranes consistently provided high quality (λ260:280 >1.8) RNA with no significant difference between the TZ and RN methods (p=NS).

Table 8-1: Comparison of RNA quality and yield obtained via various cell collection and processing methods

	MP + TZ	MP+RN (1mL)	PES + TZ	PES+RN (1mL)
Number of Eyes Providing Data	6/6	6/6	6/6	6/6
Range of λ260:280	1.7 - 2.2	2.0-2.3	1.7 - 2.2	1.8-2.2
Mean Yield (μg)	14.9	18.1	9.6	11.1
Range of Yields (μg)	8.1 - 25.8	12.7-27.5	4.0 – 19.7	6.0-24

The integrity and size distribution of total RNA purified with the above mentioned kits were verified by denaturing formaldehyde agarose gel electrophoresis and stained with ethidium bromide. The respective ribosomal bands (Figure 8-3) appeared as sharp bands on the stained gel. The 28S ribosomal RNA bands appeared with intensity approximately twice that of the 18S RNA band. No lane had smeary or small sized RNAs showing no sample degradation during preparation.

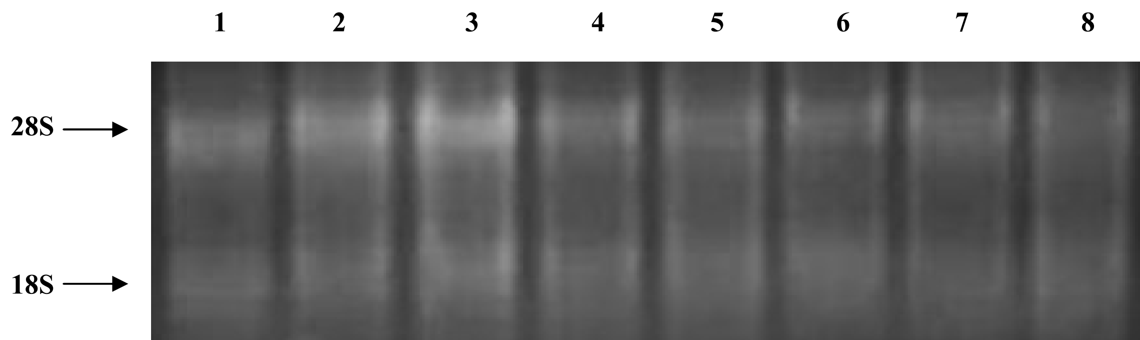

Figure 8-3: **RNA integrity gel**

5 μg of total RNA isolated from eight different conjunctival cell collections (lanes 1-8) was analyzed on a denaturing 1% formaldehyde agarose gel stained with ethidium bromide.

Figure 8-4 represents the results of RT-PCR amplification of MUC1 and GAPDH (glyceraldehyde- 3-phosphate dehydrogenase - housekeeping gene) mRNA. Lanes 2, 4, 7, 9 represent negative controls confirming the lack of DNA contamination in the samples.

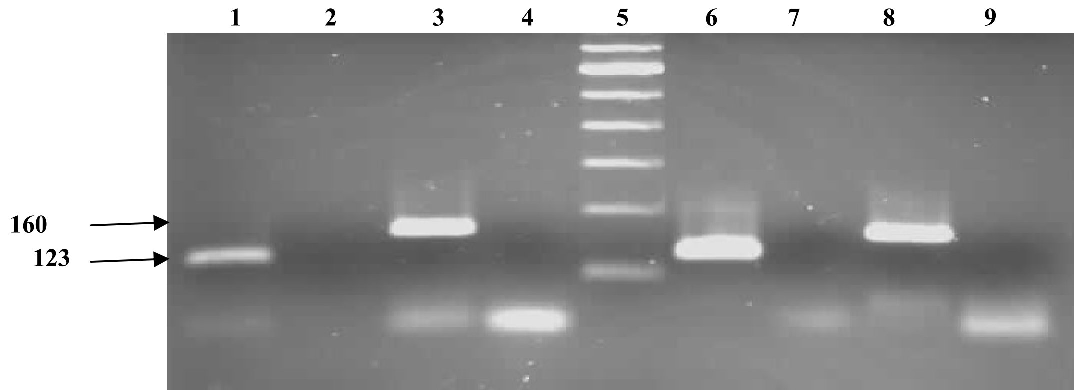

Figure 8-4: RT-PCR amplification of MUC1 and GAPDH mRNA

Amplification of mRNAs coding for MUC1 (123 bp) and GAPDH (160 bp) from two different samples of total RNA (lanes 1 – 4 and 6 – 9) isolated using the RN method. Lanes 2, 4, 7 and 9 represent negative controls, where RT reactions were run in the absence of reverse transcriptase enzyme. Lane 5 = molecular weight ladder.

Figure 8-5a represents the impression cytology of normal human conjunctiva stained using Haematoxylin and Eosin (appendix G). The membrane to the left in Figure 5a represents a PES membrane and the membrane to the right in Figure 8-5a represents a MP membrane. When these stained membranes were evaluated by light microscopy under 40X magnification, both membranes showed thousands of epithelial cells adhered to the membrane. Figure 8-5 b and c represents PES and MP membrane respectively.

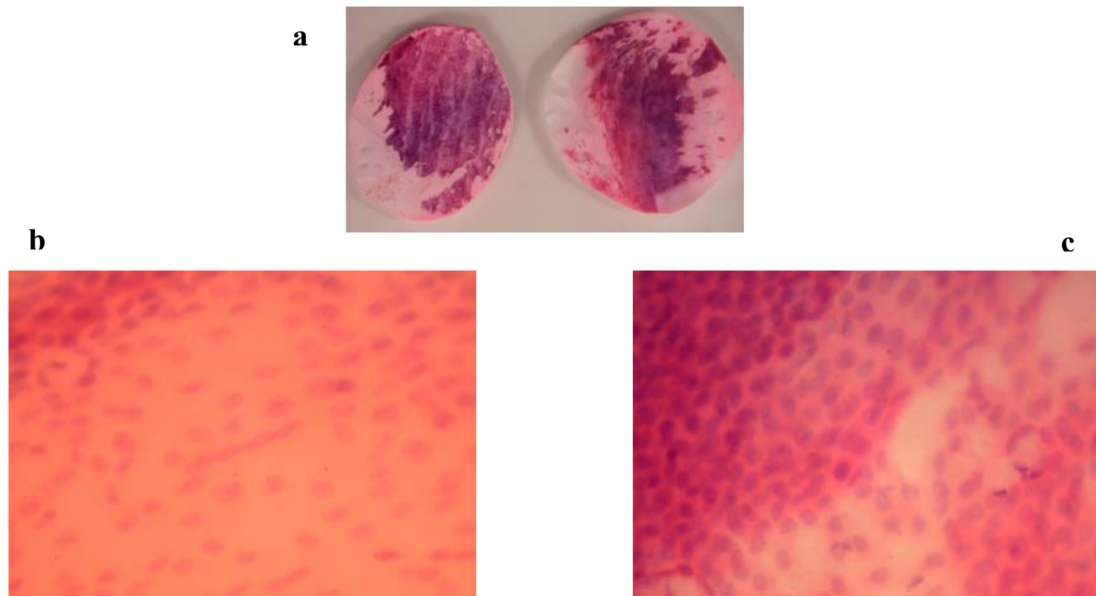

Figure 8-5: Impression cytology of normal conjunctiva (Hematoxylin and Eosin stain-H&E)

(a) PES membrane to the left and MP membrane to the right (b) PES membrane stained in H&E stain under 40X magnification (c) MP membrane stained in H&E stain under 40X magnification

When the mean yield of protein extracted from MP membrane was assessed, the four different procedures had no significant difference (p=NS) in the amount of protein extracted (Figure 8-6). Error bars represent standard deviation. However, the mean yield of protein obtained using flash freezing in EB+PI was higher than the rest of the techniques that were examined (mean yield = 9.67 ± 5.64) though not statistically significant.

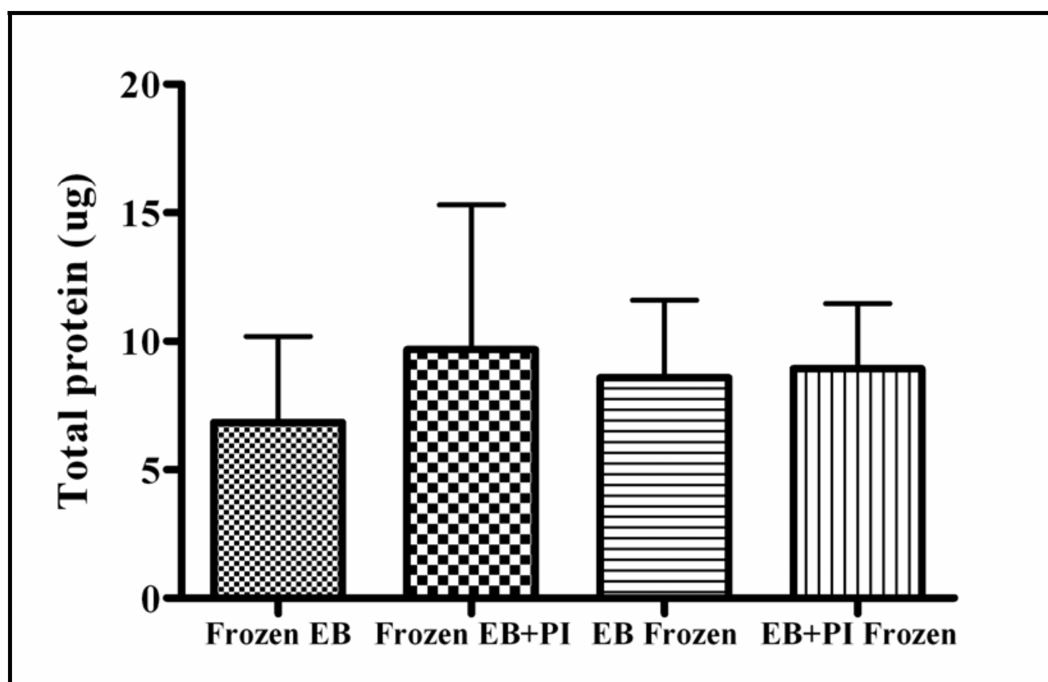

Figure 8-6: Mean yield of protein using EB and EB + PI

This graph represents the average amount of protein in µg found in 10µL of CIC samples. Error bars represent standard deviation.

Figure 8-7a represents a LOX2 Western blot performed on CIC derived protein samples. Lanes 1-4 are Human Recombinant 15-LOX Form-2 Standard (Lane 1 = 2.89, Lane 2 = 1.44, Lane 3 = 0.72, Lane 4 = 0.36 ng/µL); Lanes 5 - 8 are CIC samples taken from the same subject (Lane 5 = Left Superior; Lane 6 = Left Temporal, Lane 7 = Right Superior; Lane 8 = Right Temporal regions of conjunctiva). Figure 8-7b represents a regression curve that was plotted by graphing applied concentration of LOX 2 standard against the optical density of the resulting band immunoreactivity. Total LOX 2 concentration was quantified by extrapolation from this curve (Lane 5 = 0.91, Lane 6 = 0.58, Lane 7 = 2.38, Lane 8 = 6.58 ng/µg of Total Protein).

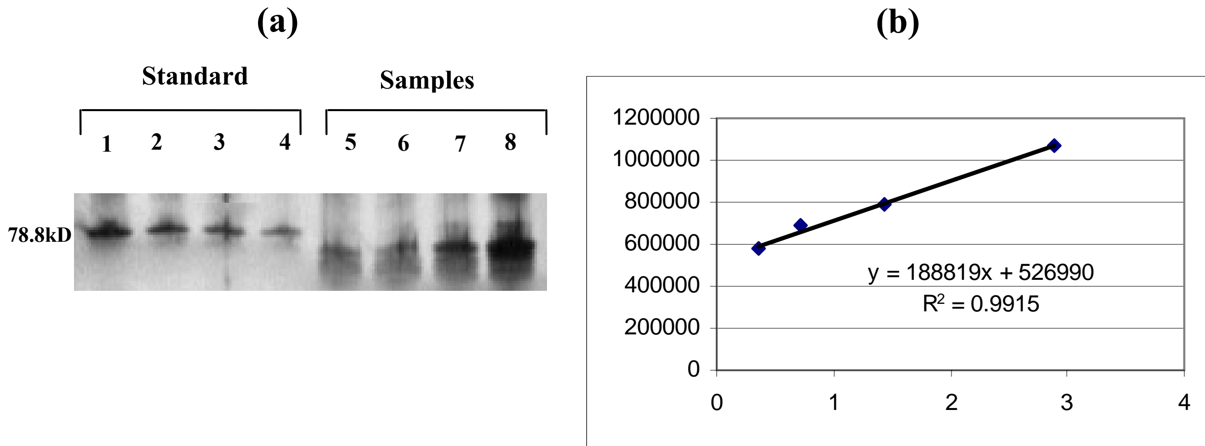

Figure 8-7: Western blot and regression analysis for LOX 2 quantification

(a) An example of a LOX 2 Western blot from CIC derived protein samples. Lanes 1-4 are Human Recombinant 15-LOX Form-2 Standard (Lane 1 = 2.89, Lane 2 = 1.44, Lane 3 = 0.72, Lane 4 = 0.36 ng/μL); Lanes 5 - 8 are CIC samples taken from the same subject (Lane 5 = Left Superior; Lane 6 = Left Temporal, Lane 7 = Right Superior; Lane 8 = Right Temporal regions of conjunctiva). (b) A regression curve (graphing applied concentration of LOX 2 standard against the optical density of the resulting band immunoreactivity). Total LOX 2 concentration was quantified by extrapolation from this curve (Lane 5 = 0.91, Lane 6 = 0.58, Lane 7 = 2.38, Lane 8 = 6.58 ng/μg of Total Protein).

Figure 8-8 represents the average amount of LOX 2 in ng found in 1μg of total protein from the superior or temporal CIC samples. There was no significant difference (p=NS) in the extracted protein from the two regions. Error bars represent standard deviation.

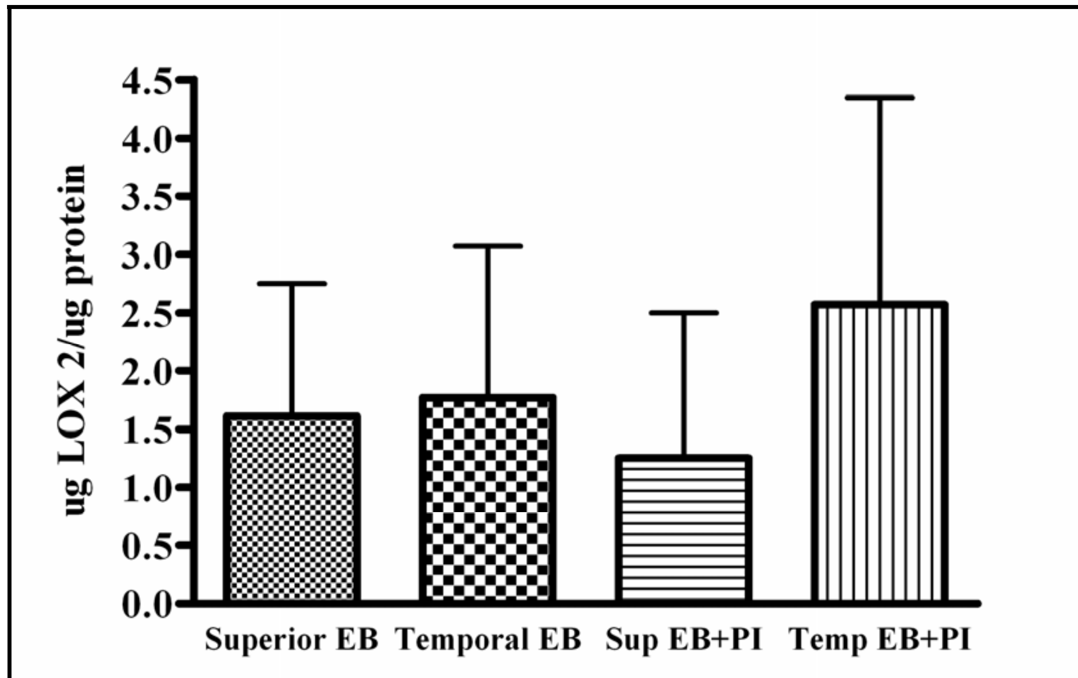

Figure 8-8: Mean yield of LOX 2

This graph represents the average amount of LOX 2 in ng found in 1µg of total protein from the superior or temporal CIC samples. Error bars represent standard deviation.

8.6 Discussion

In this chapter, optimized methods for isolation of both total RNA and total protein isolation from conjunctival epithelial cells collected via impression cytology (CIC) were presented. In first section, details describing the use of two different filter paper membranes that are routinely used for CIC namely PES and MP, were used in conjunction with two different kits that are commercially available to isolate RNA form the cells. The second section explains the two techniques to isolate total protein from MP membrane.

Studies characterizing the conjunctival surface have often used tissue samples and biopsies obtained during cataract surgery,[27] conjunctival tissue collected from eye bank donors[30] or have used animal models[31] to describe conjunctival morphology[32] or histological characteristics of the ocular surface epithelia.[33] These procedures are obviously highly invasive and impractical to undertake on a routine basis. One of the main advantages of CIC is that it can be performed in a routine clinical setting, enabling clinicians and researchers to collect viable epithelial cells, which can be subsequently analyzed using an endless list of techniques, including microscopy,[4, 12, 34] flow cytometry,[35, 36] Western blotting[37] and Reverse Transcription - Polymerase Chain Reaction (RT-PCR) [7, 16, 17, 22] Of all the various different membranes[1-3, 9, 38, 39] and devices[40] available to perform impression cytology; cellulose acetate membranes have attracted the most use.[3, 12]

This study demonstrated that MP membranes processed with either the TZ or RN methods are equally efficient for the isolation of high quality RNA from conjunctival cells collected *in situ* via CIC. The phase separation method (TZ) is one of the least

expensive procedures[20] and is also a state of the art protocol if microRNA processing is required. During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. However, time consuming and tedious methods such as CsCl step gradient ultracentrifugation and alcohol precipitation steps, or methods involving the use of toxic substances such as phenol and /chloroform, are replaced by the RNeasy procedure. The advantage of the RN technique is that it can isolate all RNA molecules longer than 200 nucleotides, although it has a maximum loading capacity of 100 µg for each mini column. The RNA obtained using this technique is of high quality and integrity and can be used for downstream applications such as cDNA synthesis, RT-PCR, and gene array analysis. From a "user friendly" view point, the adoption of the RN method due to enhanced speed as well as on-column isolation and DNase-digestion is recommended.

In addition, this study describes a method based on CIC to collect high quality protein from the human ocular surface. It should be noted that this is the first known account for isolation of total protein. Results suggested that there was no significant difference in the amount of protein extracted from membranes processed via the various methods assessed. All methods provided an adequate yield of intact protein as evidenced by successful Western blotting of a constitutively expressed protein (LOX2). In a clinical research set up, CIC followed by immediate flash freezing is useful. Thus, from a "user friendly" view point, we recommend the adoption of the flash freezing method post CIC and that EB+PI be used to extract protein.

8.7 Conclusion

CIC has been used in many clinical studies and has attracted the attention of many researchers and clinicians. Due to its simplicity, ease of use and ability to facilitate both gene and protein analysis as demonstrated in this work, one can anticipate the increased utility of CIC in the years ahead as we strive to more thoroughly characterize the ocular surface.

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9 Expression of MUC1 and MUC16 in tears and conjunctival epithelial cells collected from postmenopausal women experiencing symptoms of dry eye

9.1 Abstract

Purpose: To quantify the expression of MUC1 and MUC16 mRNA and protein in a group of symptomatic dry-eyed postmenopausal women (PMW) compared to asymptomatic controls and to investigate the potential relationship between mucin expression and tear film breakup time.

Methods: 83 healthy PMW (>50 years of age) were categorized as being symptomatic of dry eye (DE) or asymptomatic (NDE)) based on their responses to the Allergan Ocular Surface Disease Index© (OSDI) questionnaire. Non-invasive tear breakup time (NITBUT) was evaluated using the ALCON Eyemap®. Tears were collected from the inferior tear meniscus using a disposable glass capillary tube and an eye wash method. Conjunctival epithelial cells were collected via impression cytology and from these specimens both total RNA and total protein were isolated. Expression of MUC1 and MUC16 mRNA was assessed via real time PCR. Expression of both membrane-bound and soluble MUC1 and MUC16 were quantified via Western blotting.

Results: OSDI responses identified 44 symptomatic (age = 63.6±9.4 yrs) and 39 asymptomatic (age = 59.5±6.6 yrs) participants. The DE group exhibited a significantly shorter NITBUT (DE = 5.3 ± 1.5 sec; NDE = 7.0 ± 2.7 sec; p=0.005). No difference in

MUC1 or MUC16 mRNA or protein (soluble or membrane bound) expression was found between DE and NDE. Weak correlations were found between the NITBUT values compared with MUC 1 and MUC16 mRNA and protein expression.

Conclusions: No difference was found in the expression of either MUC1 or MUC16 protein or mRNA expression between symptomatic PMW and asymptomatic controls. Symptomatic women did differ from controls with respect to significantly reduced NITBUT. NITBUT values do not appear to be associated with MUC1 and MUC16 expression (protein or mRNA). Further research is required to investigate the potential use of biomarkers such as MUC1 and MUC16 in the characterization of dry eye disease.

9.2 Introduction

Numerous compositional models of the tear film have been proposed. The first description by Wolff[1] in 1946, presented a three-layered tear film, consisting of an anterior lipid layer, middle aqueous layer and inner mucin layer. As additional information became available, this model evolved to accommodate the possibility of soluble mucins in the aqueous layer, decreasing in concentration towards the lipid layer.[2] The present concept is that the tear film is a bilayered structure, consisting of an aqueous/mucinous phase and an outermost multi-layered lipid phase.[3]

Of the various components of the tear film, mucins are thought to play a key role in the retention of water and other tear fluid components on the ocular surface, facilitating a healthy, wet ocular surface. Earlier, mucins were only thought to be secreted by goblet cells. However, studies have now shown that membrane-associated mucins also exist.[4-7] To-date, at least twenty different mucin subtypes have been characterized.[6, 8-16] Both secreted (MUC2, MUC5AC, MUC5B, MUC7) and membrane bound (MUC1, MUC4, MUC16) forms have been reported to be expressed by ocular surface epithelia.[17, 18] Of the mucins identified on the ocular surface, two soluble (MUC2 and MUC5AC) and three membrane-bound (MUC1 and MUC 4, MUC 16) forms are considered “critical” for the maintenance of a “normal” tear film. [17] MUC2 and MUC5B are present in very low quantities.[17, 19]

Alternative forms of MUC1[20] and MUC16[19] exist,[21, 22] as recently illustrated by Hori et al,[23] who described soluble MUC16 in the tears. The alternative forms of MUC1 and MUC 16 lack the cytoplasmic tail portion of the protein and thus are secreted or shed into the tear film, as opposed to being anchored into epithelial cell membranes. Whether

these secreted species are present in all tear samples and/or whether their concentrations differ between subjects complaining of dry eye compared to asymptomatic subjects has yet to be investigated. The specific functions of secreted mucins are not known, although, evidence does suggest that MUC16 is an important hydrophilic molecule involved in the maintenance of a healthy ocular surface.

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. To date, the primary focus has been MUC5AC.[24] The majority of these studies have focussed on Sjogren's syndrome, young adults with dry eye complaints, contact lens wearers, allergic conjunctivitis or non-human (rodent) studies.[24-30]

The results of large epidemiological studies[31] conducted in the United States clearly suggest that the prevalence of dry eye is greater in women than in men and that approximately 3.2 million women and 1.6 million men aged 50 years or older suffer from moderate to severe dry eye and women frequently consult clinicians with symptoms of ocular dryness and discomfort.[32, 33] To date, no work has focused on whether post menopausal women (PMW) complaining of dry eye disease show any difference in the expression of ocular muicns. Such information is needed to help understand the role played by various mucins in dry eye disease and would help to guide the development of mucin secretagogues intended for the therapeutic treatment of dry eye.

As highlighted in earlier chapters of this thesis, due to the complicating factors in the diagnosis and management of dry eye, an enhanced biochemical understanding of the pathophysiological / biochemical processes underlying dry eye would enable both the

proper utilization and continued development of specific and sensitive diagnostic tests to facilitate objective reproducible and standardized diagnostic tests and methods to evaluate treatment efficacy.

In light of the above, the aim of this study was to quantify the expression of MUC1 and MUC16 protein (soluble and membrane bound) and mRNA in a group of symptomatic dry eyed PMW compared to asymptomatic controls and also to investigate the potential relationship between mucin expression and tear film break up time.

9.3 Methods

9.3.1 Participants

Informed consent was obtained from all participants following explanation of the purpose of the study and its various procedures, prior to participation in the study. A case history and complete ocular surface examination was performed to determine participant eligibility. Participants on hormone replacement therapy (HRT) were excluded, as were contact lens wearers and participants receiving any topical ocular medication or systemic medication known to exacerbate dry eye. Participants with a prior history of blepharitis and/or active blepharitis at the time of recruitment were also excluded from the study. For the purpose of this study, "postmenopausal" was defined as no menses for at least one year, not associated with hysterectomy. Eighty three healthy PMW greater than 50 years of age were recruited.

9.3.2 Subjective symptoms

Participants completed the Allergan Ocular Surface Disease Index© (OSDI) questionnaire[34] and were categorized as being symptomatic (44 individuals; age = 63.6±9.4 yrs) or asymptomatic (39 individuals; age = 59.5±6.6 yrs) of dry eye, based on their response to OSDI, as described in chapter 4 section 4.3 of this thesis and in detail elsewhere.[34] This study was conducted in two sections. The first section was named post menopausal women study 1 (PM1) that consisted of 37 participants. Post menopausal women study 2 (PM2) consisted of 46 participants.

9.3.3 Objective measurements

Tear stability was assessed by performing non invasive tear breakup time (NITBUT) using the ALCON Eyemap® model EH-290 topography system (ALCON, Inc., Forth Worth, Texas, USA). This is explained in detail in chapter 3, section 3.4.

9.3.4 Analytical techniques

9.3.4.1 Reagents and materials

A SE600 vertical gel unit was purchased from E Biosciences (Amersham, San Diego, California, USA). 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 transfer buffer were purchased from EMD, USA. Molecular weight standards (Himark prestained protein standard) were purchased from Invitrogen (Carlsbad, California, USA). ECL-Plus™ kits were purchased from GE Healthcare (Baie d'Urfe, QC, Canada). Immuno-Blot® PVDF (polyvinylidene difluoride) membrane and DC Protein Assay Kit® were purchased from BioRad Laboratories (Mississauga, ON, Canada). Mouse

monoclonal anti-human MUC 1 antibody (DF3) was purchased from Signet (Dedham, MA, USA), 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). RNeasy® Mini kit was purchased from Qiagen Inc. (Mississauga, ON, Canada). Beckman DU530 Life Science UV/Visible Spectrophotometer from Beckman Coulter (Fullerton, CA, USA) and Taqman® Universal PCR Master Mix and 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) were used in the study.

9.3.4.2 Capillary tear collection

Using a graduated disposable 5 μl microcapillary tube (Wiretol-Micropipettes, Drummond Scientific Co., Broomall, PA, USA) up to 5 μl of tears / eye were collected from the inferior temporal tear meniscus of the PM1 and PM2 participants. Further details are presented in chapter 3, section 3.6.

9.3.4.3 Eye wash tear collection

A second method of tear collection, using an eye wash method, was undertaken on only PM2 participants, as described elsewhere.[24] Further details are presented in chapter 3, section 3.10. The two collections were separated by 15 – 25 minutes to allow for tear film regeneration.

9.3.4.4 Conjunctival impression cytology (CIC)

Conjunctival epithelial cells were collected from all volunteers via impression cytology using 10mm Millipore (MP) membranes placed on both the superior and

temporal conjunctiva of both eyes. The left eye and right eye samples were kept separate. The CIC samples from the right eye were used for mRNA expression work. Samples from the left eye were used for analysis of MUC1 and MUC16 protein. Preparation of CIC membranes and the entire CIC procedure is described in detail in chapter 3, section 3.11. CIC samples were collected from both PM1 and PM2 participants.

9.3.4.5 Electrophoresis and immunoblotting

Total protein was isolated from epithelial cells (left eye) collected using 50µl of extraction buffer (50 mM Tris, 2% SDS, 1X Protease Inhibitor™, (PI) (Roche Diagnostics, Indianapolis, Indiana, USA). Total protein in 5 µL of cell lysate, 0.5 µL of capillary tear samples or 5 µL of the eyewash samples were quantified using BioRad's DC™ protein assay. Step-wise procedures are detailed in chapter 3, sections 3.13.1 and 3.13.2 and appendices D & E.

Protein samples were subjected to agarose gel electrophoresis using a SE600 vertical gel unit, then transferred to nitrocellulose membranes in a vacuum blotter. A titration of MUC16 standard antigen (CA125, Biodesign) or MUC 1 standard antigen (CA15-3, Biodesign) was run on each gel to normalize data and facilitate semi-quantitation of samples, through linear regression analysis. For MUC1 identification, 6 µg/lane of total protein for tears and 20µg/lane of total protein for IC samples was loaded per lane. For MUC16, 4.0 µg/lane of total protein for tears and 5.0 µg/lane of total protein for IC samples was loaded per lane. MUC1 was identified using a mouse monoclonal DF3 primary antibody, followed by an anti-mouse IgG secondary antibody. MUC 16 was identified using a mouse monoclonal OC 125 primary antibody, followed by goat anti-mouse IgG secondary antibody. This is explained in detail in methods

chapter 3, section 3.13.4. Immunoreactivity was visualized with ECL Plus® chemiluminescent substrate (GE Health Care) and the optical densities of the resulting bands were quantified from digitized images created with a Molecular® Dynamics Storm™ 840 Imager using ImageQuant™ 5.1.

9.3.4.6 RNA isolation and reverse transcription

Total RNA was isolated from impression cytology samples using RNeasy™ Mini (RN) kit (Qiagen), according to the manufacturer's protocol, with several modifications. This is explained in detail in Appendix G. RNA quantity and quality was assessed by measuring the optical density using a Beckman DU530 Life Science UV/Visible Spectrophotometer at 260nm and 280nm. DNA was synthesized from 8μL 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 instruction.

9.3.4.7 Real time- qPCR

Relative expression of genes of interest was performed in multiplex PCR reactions containing target and endogenous control oligonucleotide primers in the presence of gene-specific dye-labeled Taqman probes (Table 9- 1). Step wise procedures are discussed in chapter 3, section 3.13.8.

Table 9-1: Sequence of primers and probes used for gene amplification in Real Time RT-PCR

Gene	Forward Primer	Reverse Primer	Taqman Probe
MUC1	CTGGTCTGTGTTC TGGTTGC	CCACTGCTGGGTT TGTGTAA	6FAM-GAAAGAACTACGGGCA GCTG
MUC16	ACCCAGCTGCAG AACTTCA	GGTAGTAGCCTGG GCACTGT	6FAM-GCGGAAGAAGGAAGGA GAAT
GAPDH	GAAGGTGAAGGT CGGAGTCA	GACAAGCTTCCCG TTCTGAG	VIC-CAATGACCCCTTCATTG ACC

Conventional RT-PCR experiments were performed to confirm the identity of the PCR products. Following 40 cycles of cDNA amplification using the MUC1 and MUC16 primers detailed above, unique bands corresponding to the predicted size for MUC1 and MUC16 was obtained. PCR products were electrophoresed in 1.0% agarose gels containing ethidium bromide. The bands in the agarose gel were excised and extracted for sequencing analysis (Alcon Research Ltd, USA) to verify the identity of the MUC1 and MUC16 PCR products. For real-time qPCR, the expression levels of mRNA were normalized by the median expression of the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase).

9.3.5 Statistical analysis

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

used for comparison of OSDI questionnaire and NITBUT data. 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 $p<0.05$ ($\alpha = 0.05$). Pearson correlations between NITBUT and mucin expression were calculated in Statistica Ver7.1 (StatSoft Inc., Tulsa, OK, USA).

9.4 Results

The mean age of the participants was 63.6 ± 9.4 yrs in the symptomatic group (n=44) and 59.5 ± 6.6 yrs in the asymptomatic group (n=39). Of the 83 participants enrolled, only one participant reported severe dry eye symptoms. So as to work with two defined dry eye sub-groups (mild and moderate), this "severe" subject was excluded from analysis, resulting in 43 participants in the dry eye group.

9.4.1 Subjective symptoms

In this study, the presence and severity of dry eye was determined based on symptoms only, as quantified by total OSDI score, using the following criteria: control (non-dry eye, NDE) OSDI score = 0-12; mild dry eye OSDI score = 13-22 and moderate dry eye OSDI score=23-32 (see Table 9-2). Based on this criterion, 39 subjects were defined as controls. 16 subjects presented with mild dry eye and the remainder (n=27) were classified as moderate. Mean ages (mean ± SD) of the control, mild and moderate groups were 59.6 ± 6.6, 63.5 ± 10.5 and 63.4 ± 8.5 years respectively. There was no significant difference between the ages of the three groups (p=0.07). The total OSDI scores for the two dry eyed groups were significantly higher compared to the NDE group

(p<0.0001), as was the total score of the moderate group compared to the mild group (p=0.01). Analysis of the individual OSDI sub scores revealed a significantly elevated score for the mild and moderate DE groups in each category compared to the NDE group, with the exception of the Vision Related Function score, where there was no distinction between NDE and mild dry eye. Mild and moderate dry eye sub scores were statistically similar, with the exception of the Vision Related Function score, which was significantly elevated in the moderate group (p<0.01).

Table 9-2: Summary of Ocular Surface Disease Index© Scores

OSDI Score	Overall			Subcategory				
	NDE (n=39)	DE (n=43)	p (α =0.05) (NDE vs DE)	Mild DE (n=16)	Moderate DE (n=27)	p (α =0.05) (NDE vs Mild)	p (α =0.05) (NDE vs Moderate)	p (α =0.05) (Mild vs Moderate)
Total Score	7.43 ± 7.71	24.87 ± 13.89	**<0.001***	18.37 ± 9.29	28.31 ± 13.02	**<0.001***	**<0.001***	**0.01***
Ocular Symptoms	7.56 ± 8.42	23.98 ± 19.21	**<0.001***	17.35 ± 13.59	26.73 ± 24.49	**0.002***	**<0.001***	0.10
Vision Related Functions	7.05 ± 12.18	17.19 ± 16.42	**0.002***	8.33 ± 7.21	22.20 ± 21.42	0.11	**<0.001***	**0.003***
Environmental Triggers	8.22 ± 12.29	34.09 ± 25.97	**<0.001***	30.39 ± 21.02	36.86 ± 27.21	**<0.001***	**<0.001***	0.68

Figure 1 reports the NITBUT determined by the corneal topographer. NITBUT was significantly reduced in both mild (p=0.02) and moderate (p=0.008) DE subjects compared to the NDE group, and no difference was found between the two dry-eyed groups (p=0.88).

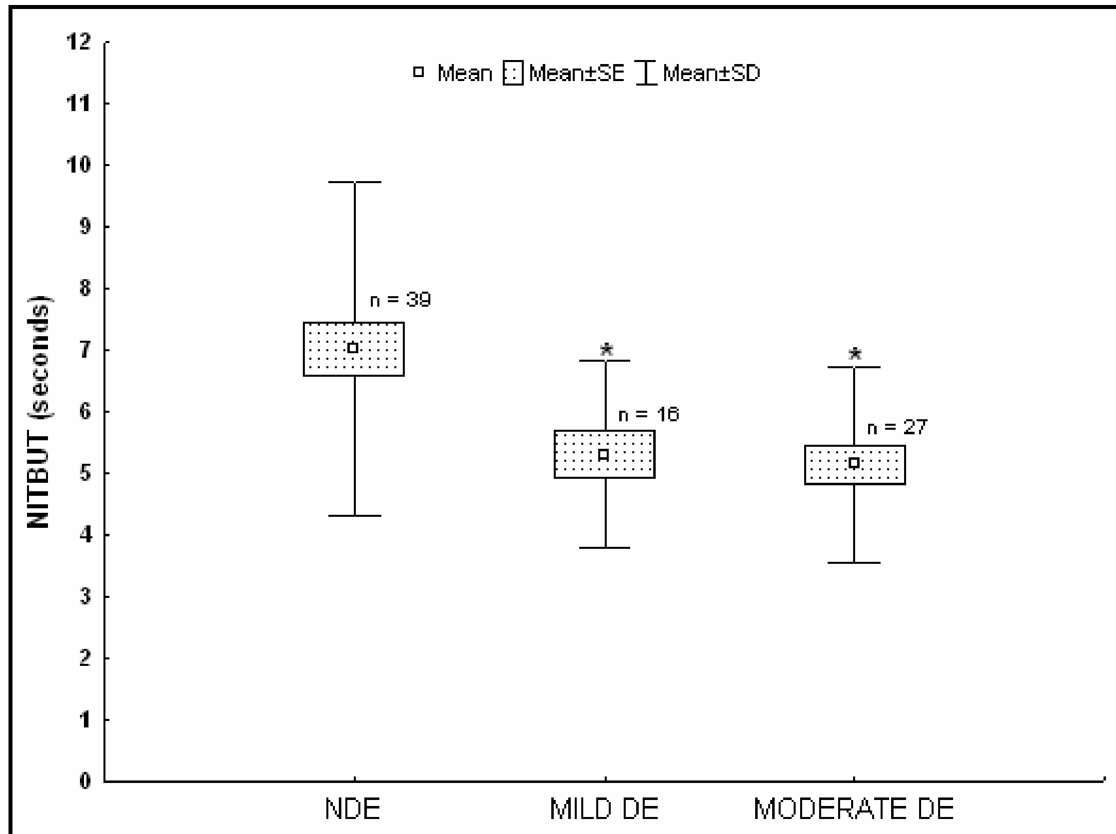

Figure 9-1: Box plots of non invasive tear film break-up measurements

Inner boxes represent mean, dotted boxes represent standard error and whiskers represent standard deviation of NITBUT measurements from non dry-eyed (NDE), mild dry eye and moderate dry eye subjects. * Represent statistically significant difference relative to NDE.

Stratification of total protein values based on dry eye symptom severity was not performed with eye wash data due to the relatively small sample size of both DE groups. Table 9-3 reports all the data of PM1. Table 9-4 represents data from PM2. All data are expressed as mean ± standard deviation. Tear collection through the eye wash method was not performed in PM1. Tear collection using the capillary technique for PM2 is not reported in this chapter, as the tears were used for lipocalin and lysozyme analysis (reported in chapter 7) and cytokine analysis (not reported in thesis).

9.4.2 Tear total protein

The total protein in the tears collected by glass capillary tube samples from PM1 are highlighted in Table 9-3. No difference in protein concentration was found comparing DE to NDE groups in tear samples ($p=0.056$). Similar findings were noted in PM2 (Table 9-2), showing no difference between eye wash methods between the two groups ($p= 0.26$). Although protein concentration was lower in tears collected using the eye wash method due to dilution with saline, no difference ($p=0.26$) was found in protein concentration between the NDE (1.91 ± 0.94 μg/μL) and pooled DE groups (1.61 ± 0.81 μg/μL).

9.4.3 Expression of MUC1 protein in tears and conjunctival epithelial cells

Quantification of all chemiluminescent signals >150 kDa, revealed no significant differences in either tear or IC MUC1 protein expression between DE and NDE as calculated by extrapolation from linear regression plots constructed from CA15-3.

9.4.4 Expression of MUC16 protein in tears and conjunctival epithelial cells

Electrophoretic patterns of all samples suggested numerous isoforms of both soluble and membrane bound MUC16 exist. Quantification of all chemiluminescent signals >150 kDa, revealed no significant differences in either membrane bound or soluble mucin expression between DE and NDE for PM1 as calculated by extrapolation from linear regression plots constructed from CA125 (Figure 9-3). MUC16 expression in eyewash samples showed no difference in the DE and NDE groups as shown in Table 9-4. Example of a Western blot from IC samples is shown in Figure 9-2.

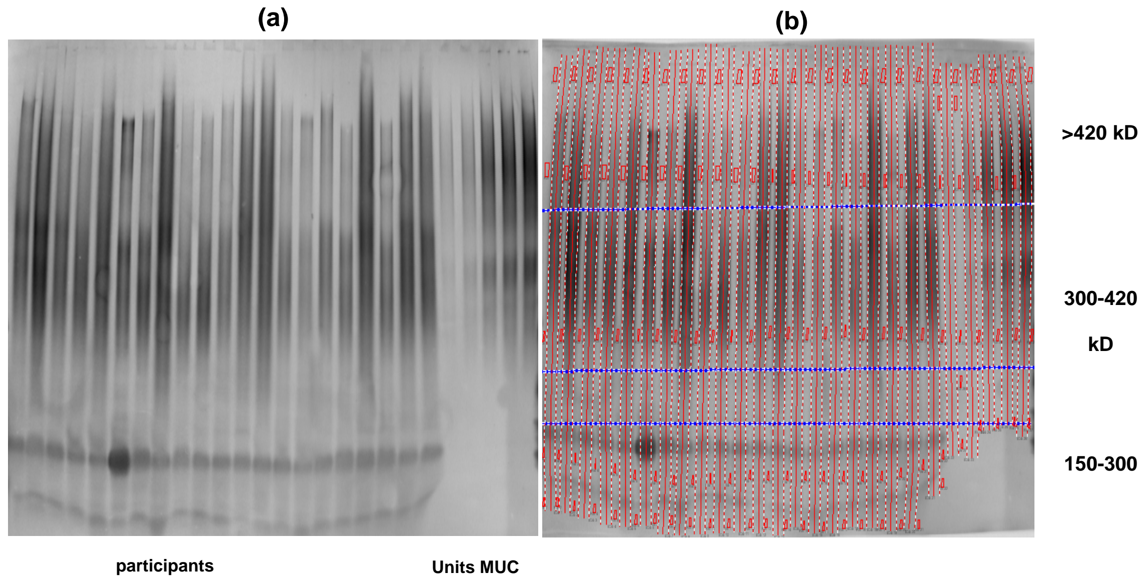

Figure 9-2: Western blot for MUC16

(a) An example of membrane bound MUC 16 Western blot from IC protein samples. Last 5 lanes are the MUC 16 Standard, CA 125, 5-70 units (b) The same blot showing how the lanes were divided and scored for blot intensity.

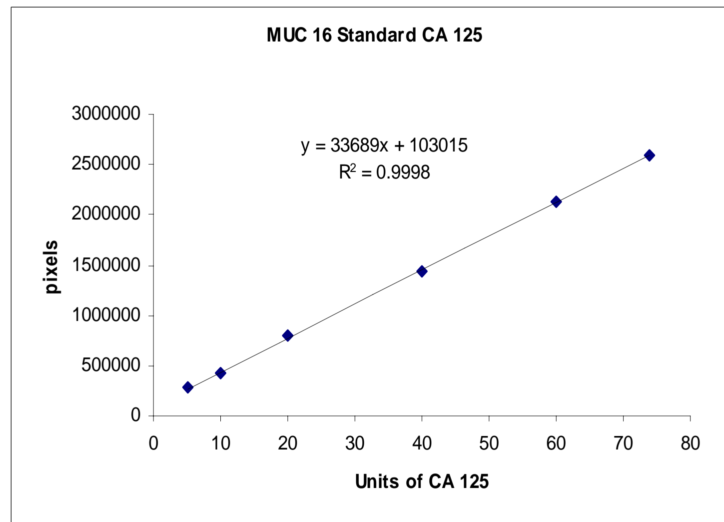

Figure 9-3: Regression analysis for MUC 16 quantification

A regression curve was created by graphing applied concentration of MUC 16 standard against the optical density of the resulting band immunoreactivity. Total MUC 16 concentration was quantified by extrapolation from this curve.

Table 9-3: Summary of PM1 study biomarker data

ID 1 to 39 (PM1 study)				
Biomarker	Sample type	Normal	Dry eye	p value
Total Protein (µg)	Capillary tears	6.6 ±2.6 (n=19)	6.7 ± 2.1 (n=17)	0.88
Tear Film MUC1* (AU)	Capillary tears	0.04 ± 0.08 (n=19)	0.06 ± 0.09 (n=17)	0.4
Tear Film MUC16** (AU)	Capillary tears	2.66 ± 1.29 (n=15)	2.70 ± 1.83 (n=12)	0.95
Membrane Bound[+] MUC1* (AU)	IC	0.014 ± 0.013 (n=19)	0.015 ± 0.014 (n=18)	0.84
Membrane Bound[+] MUC16** (AU)	IC	15.17± 8.04 (n=19)	18.15 ±10.34 (n=18)	0.35
MUC1 mRNA (RQ)	IC	0.70±0.06 (n=18)	0.93±0.24 (n=16)	0.077
MUC16 mRNA (RQ)	IC	0.63.±0.30 (n=18)	1.39±1.10 (n=16)	0.01[§]

*** MUC 1 ≤ 250 kDa; ** MUC 16 ≤ 350 kDa; + Denotes Membrane bound fraction of MUC1 or 16 derived from epithelial cells collected via conjunctival impression cytology; IC = Impression cytology samples; AU = arbitrary units based on extrapolation from internal standard; RQ = mean value for gene of interest normalized to expression of GAPDH mRNA; n/a = not applicable; [§] = significant difference**

9.4.5 Expression of MUC1 mRNA in conjunctival epithelial cells

Real time PCR results showed that the levels of the MUC1 remained unaltered in both groups in PM1 and PM2 (p=0.077 and 0.27 respectively). The internal standard that allows pooling the samples between studies 1 and 2 failed (a single sample was

designated and used as a "reference” and the amplification of that sample failed in PM2); hence the results from MUC1 from PM1 and 2 are reported individually.

9.4.6 Expression of MUC16 mRNA in conjunctival epithelial cells

PM1 (n= 34) showed a significant increase in the MUC16 mRNA expression in the DE group (p=0.01). Upon addition of data from PM2, (n=78), there was no significant difference in the MUC16 expression (p=0.06).

Table 9-4: Summary of PM2 study biomarker data

ID 40 to 86 (PM2 study)				
Biomarker	Sample type			
		Normal	Dry eye	p value
Total Protein	EW	1.91±0.94 (n=20)	1.61±0.81 (n=24)	0.26
Total Protein	Capillary Tears	8.41±3.7 (n=20)	6.80±3.31 (n=20)	0.14
MUC1*	EW	0.37±0.34 (n=14)	0.56±0.47 (n=15)	0.25
MUC16**	EW	0.37±0.33 (n=16)	0.54±0.39(n=21)	0.149
Membrane Bound+ MUC1*(AU)	IC	1.76±0.74 (n=19)	1.79±0.78 (n=22)	0.909
Membrane Bound+ MUC16** (AU)	IC	0.57±0.21 (n=20)	0.62±0.28 (n=24)	0.476
MUC1 mRNA (RQ)	IC	0.63±0.19 (n=20)	0.79±0.22 (n=24)	0.27
MUC16 mRNA (RQ)	IC	0.35±0.11 (n=20)	0.57±0.72 (n=24)	0.28
MUC16 mRNA (RQ) TOTAL (PM1 and 2)	IC	0.36±0.21 (n=38)	0.67±0.83 (n=40)	0.06

IC = Impression cytology samples; EW = Eye wash tear samples; * MUC 1 ≤ 250 kDa ** MUC 16 ≤ 350 kDa; + Denotes Membrane bound fraction of MUC1 or 16 derived from epithelial cells collected via conjunctival impression cytology; RQ = mean value for gene of interest normalized to expression of GAPDH mRNA; AU = arbitrary units based on extrapolation from internal standard; ND- not done; n/a = not applicable

9.4.7 Correlation between mucin expression and NITBUT

Correlation analysis between NITBUT and mucin expression showed weak and insignificant correlation for both PM1 and PM2, as highlighted in Tables 9-5 and 9-6. MUC16 mRNA expression and NITBUT showed a weak but significant correlation for PM1 only.

Table 9-5: Summary of correlations between NITBUT and mucin expression in PM1 study

ID 1 to 39 (PM1 study)		
Biomarker	**NITBUT**	**p value**
Tear Film MUC1	-0.23	0.16
Tear Film MUC16	-0.21	0.30
Membrane Bound MUC1	-0.10	0.60
Membrane Bound MUC16	-0.21	0.20
MUC1 mRNA	-0.13	0.46
MUC16 mRNA	-0.37	0.04*

* significant correlation

Table 9-6: Summary of correlations between NITBUT and mucin expression in PM2 study

ID 40 to 86 (PM2 study)		
Biomarker	**NITBUT**	**p value**
Eye wash MUC1	-0.17	0.37
Eye wash MUC16	-0.20	0.37
Membrane Bound MUC1	0.10	0.59
Membrane Bound MUC16	-0.30	0.84
MUC1 mRNA	-0.20	0.25
MUC16 mRNA	-0.16	0.30

9.5 Discussion

In this study we quantified the expression of MUC1 and MUC16 mRNA and protein in a group of symptomatic dry-eyed PMW compared to asymptomatic controls. Such information is needed to help understand the role played by mucins in dry eye disease and will help to guide the development of mucin secretagogues intended for the therapeutic treatment of dry eye. We also have attempted to explore the potential relationship between mucin expression and tear film breakup time.

We chose to stratify subjects solely on symptoms, as quantified by the OSDI questionnaire. It is a validated questionnaire recommended by the DEWS 2007 report and it is one way of potentially classifying participants based on symptom scores. Based on the OSDI criteria, we enrolled three statistically distinct groups: those without symptoms

of dry eye and those with either mild or moderate dry eye. Review of objective measurements suggested that both tear secretion and NITBUT were significantly reduced in moderately symptomatic dry-eyed subjects relative to controls. Additionally, mildly symptomatic dry-eyed subjects presented significantly reduced NITBUT relative to controls.

Power analysis to determine sample size is based on historical data. Studies on mucin biomarkers and its impact on dry eye disease have started to attract researchers' interest only in the past decade. Ocular mucin studies to date (summarized below) have utilized only small sample sizes and largely have failed to demonstrate significance. In addition, data from these studies has suggested significant variability in the concentration or expression of mucin biomarkers. Preliminary data from our lab have shown that up to 25% of recruited subjects fail to provide sufficient biological material to facilitate reliable and repeatable analysis. Thus, all taken together, it was not possible to perform mathematical power calculations, but rather we recruited a sample size larger than any study published to date.

There have been very few studies on mucin gene expression, mucin protein or mucin glycosylation in dry eye syndromes. Of these, data indicate that mucin gene expression and translation, as well as mucin post-translational processing, may be implicated in the pathophysiology of dry eye.[17] Most of the mucin expression studies conducted have focused on MUC5AC.[24, 35-38] Studies have shown the relationship between dry eye disease and decrease in the expression of goblet cell MUC5AC in KCS and Sjogren's syndrome. Such a result is expected, as goblet cells are the sole source of MUC5AC and goblet cells are reduced in conditions of dry eye.[24, 35-38] Very little is

known about the membrane spanning mucins such as MUC1 and MUC16 and their role in dry eye disease, hence, we chose to focus our efforts on these species.

Our study is novel in the fact that a unique subset of participants (PMW) was studied and that MUC1 and MUC16 were the focus. The data reported in table 9-3 and table 9-4 are different due to the fact that the standard curve used in PM1 was different from the standard curve that was used in PM2 (the analysis was performed in two different labs - each used its own scale for the standard curve). The data are expressed in arbitrary units (AU) and the relative expression (not the absolute values) was compared and statistical analysis was performed.

Few studies have shown an increased expression of mucins,[28, 39] although literature on ocular surface expression of MUC1 and MUC16 is sparse. Gipson et al,[19] using sensitive and semi-quantitative methods for the assay of mucins in tears, very recently demonstrated the presence of a soluble form of MUC16 in human tears collected using an eye wash technique similar to the technique employed in this study.[19] No relationship to dry eye was found.

Argueso et al[24] has demonstrated that the number of RNA transcripts for MUC5AC in the conjunctival epithelium and protein levels of MUC5AC in tears of patients with Sjogren's syndrome were significantly lower than in normal individuals.[24] However, the same study by Argueso et al[24] showed no significant change in MUC1 or MUC4 mRNA expressed by the stratified epithelium of the conjunctiva.[24] This study compared Sjogren's (n=11) subjects to normals (n=16). This result is in agreement with our mRNA data MUC 1 data obtained via CIC samples, showing no significant difference between the two groups evaluated in this study.

Danjo et al[26] demonstrated a significant difference in the binding pattern of an antibody against a carbonate epitope antibody (H185) carried by MUC16 to conjunctival epithelium obtained by impression cytology in normal eyes (n= 13), compared with those of patients with non-Sjogrens dry eye (n=22). However, the study was not age matched (37 vs 63 years). The results of our PM1 showed also a significant difference between the MUC16 mRNA between the two groups. However, the combined PM1 and PM2 data showed no significant difference, showing the variability in the population studied. Additional data is required to determine whether or not a true difference in MUC16 mRNA expression exists. Danjo et al[26] in the same study reported that MUC1 and MUC4 mRNA were not significantly different, although a trend toward a decrease in MUC1 mRNA was speculated.[26] Interpretation of these results is of concern because of the age range and the sample size.

The majority of the O-glycosylation sites on the transmembrane mucins are found in a highly polymorphic core region containing a variable number of tandem repeats (VNTR). Analysis of brush cytology of the conjunctiva by Imbert et al[40] has shown a decreased expression of MUC1 mRNA splice variant MUC/1A in dry eye patients (n=9) compared to controls (n=15), indicating that protein has fewer VNTRs in dry eye, which the investigators speculated may lead to poor lubrication of the ocular surface and hence ocular surface inflammation. Our data with a larger sample size did not show a variation in the MUC1 mRNA levels in the PMW dry eyed group for both PM1 or PM2.

Pflugfelder et al[27] has shown a reduction in the expression of mucosal epithelial membrane mucin in bulbar conjunctiva in Sjogrens syndrome (n=11) and non-Sjogrens syndrome dry eye (n=9) compared to normals (n=10). This study examined a small

sample size and was not age or sex matched (controls = 18-46, 5 male/5 female versus older and all female).[27] This study focused on the correlation between the goblet cell density and expression of mucosal epithelial membrane mucin in bulbar conjunctiva.

Dry eye disease is a symptom based disease and often studies fail to focus on mild and moderate forms of dry eye, which in our data set contributed to the majority of the dry eye data. Studies have shown increases in mucin expression in mild to moderate symptoms of dry eye and in allergy related studies. Hayashi et al[39] showed that mild (n = 19) and moderate (n = 17) dry eyed patients displayed increased expression of MUC1 protein as detected by KL6 antibody compared to normals (n=23) in the temporal cornea and conjunctiva. Severe dry eyed (n = 7) individuals showed a decrease in MUC1 in the cornea only and no change was noted from normals in the conjunctiva.[39] In atopic ketatoconjunctivitis (AKC), patients with significant epithelial disease and staining (n=10 eyes) showed an increase in mRNA expression for MUC1, MUC2 and MUC4 mRNA compared to normals (n=22 eyes).[28] A recent study on AKC versus vernal keratoconjunctivitis (VKC) showed higher level of tear instability, lower corneal sensitivity, up-regulation of MUC1, 2, and 4, and down regulation of MUC5AC were important differential features of the ocular surface disease in AKC (n=12), compared with VKC (n=6) and normals (n=10).[41] No tears were examined in this study.

Animal studies have also shown inconsistent results. Density gradient analysis of canine KCS tears (n=3) showed increased expression of some of the glycoprotein fractions and alteration in size and extent of mucin glycosylation.[29] Mice that have defective parasympathetic innervation of their lacrimal glands showed a reduction in the

expression of MUC1 and MUC4 mRNA in their corneal epithelium. However, no significant difference was reported.[30]

Thus, in summary, very little has been reported on MUC1 and MUC16 in dry eye and none in the population we studied. Our study was larger than any study conducted to date. Based on our data, we conclude that no difference exists in the expression of MUC1 or MUC16 mRNA or protein. Although differences in function or glycosylation cannot be ruled out, at this time, our data suggest the lack of involvement of these transmembrane mucin species in symptomatic dry eye reported by PMW.

We compared both MUC1 and MUC16 expression data with NITBUT and found weak statistically insignificant correlations. Although our work demonstrates that PMW with symptoms of dry eye had reduced tear stability, tears that were produced were biochemically within normal limits in this unique participant pool with respect to two measured ocular mucins, as well as total protein content.

All of the above mentioned studies have employed different techniques to study different species of mucins. Research in the area of "functions of mucin and the ocular surface" are relatively new and has attracted many researchers over the past 10 years. The development of sensitive assays for mucins in the tear film will permit further studies on mucin on the ocular surface and of tears from patients with ocular surface disease. Such studies will allow for improved management of these diseases.

9.6 Conclusion

We can conclude that a mixture of shed membrane-associated and secreted mucins accounts for the mucin content of tears. From our data, we can conclude that within a PMW population, expression of both MUC1 and MUC16 are unvarying, irrespective of the presence of dry eye symptoms. In addition, the concentration of either biomarker studied was not associated with tear stability. That previous studies have shown a difference in MUC1 and MUC16 concentration and/or correlation with tear dynamics may be attributed to the subgroup of dry eye subjects studied and the classification of their dry eye. This is the first comprehensive study of MUC1 and MUC16 mRNA and protein in symptomatic dry-eyed postmenopausal and our results suggest that neither would offer utility as a biomarker of dry eye.

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10 General discussion and conclusions

In this PhD project over 125 participants were screened to successfully recruit 86 participants who matched the inclusion criteria. Of these, a few were unable to complete the study, due to the reasons highlighted in the Methods section of the thesis. However, some of the participants who successfully completed the study were unable to provide sufficient tear and conjunctival cell samples to perform the required analytical tests, emphasizing the difficulty in subject recruitment and sample collection in studies of this type.

The low amounts of tear and cell sample yield often limits dry eye researchers from conducting analysis on a significant number of different dry eye biomarkers. Even though elaborate procedures have been developed for RNA isolation, working with RNA is a practical challenge. Minimal starting material and the high susceptibility of RNA to degradation were some of the major challenges faced while working with RNA samples in this study. One major draw back in this study was the inability to use vital stains to clinically diagnose dry eye. However, the presence of vital dyes in the samples may interfere with the subsequent analysis of biomarkers, especially isolation of RNA from the conjunctival epithelial cells.

Table 2-1 clearly demonstrates that none of the dry eye prevalence studies have undertaken all of the potential tests to examine the tear film and the ocular surface, and also that different studies have used different cut-off criteria for the various tests. This is primarily because of the lack of agreement amongst researchers on the diagnostic

standard for dry eye diagnosis, and also because it is far too time-consuming to conduct all the potential tests.

Chapter 4 highlights the use of dry eye questionnaires in determining the frequency and intensity of symptoms in PMW. Both the OSDI and the DEQ were valuable tools in grouping participants into those who were symptomatic and asymptomatic of dry eye. In a clinical set up, it is important to collect a range of information from a patient during the history taking. However, this task can be time consuming and the use of a questionnaire is a good alternative. One major disadvantage of questionnaires (especially in elderly individuals) is the lack of understanding of the question itself, and patients can be confused, for example, between the blurry vision induced by a dry eye condition or that induced by out-of-date spectacles. Shorter questionnaires are more "user friendly" and less time consuming, but may not provide significant information. Although lengthy questionnaires may help to gather more information, it can result in fatigue may lead to the collection of erroneous data.

Chapter 5 compared a variety of clinical tests and symptom scores between the two groups of PMW and showed a significant difference between the groups. One potential omission from this study was the inclusion of the Schirmer test, which has some disadvantages in that it may cause some reflex tearing, but it remains the "gold standard" test to confirm tear film volume and would have provided some more information on tear film dynamics in this group of PMW.

Chapter 6 demonstrated that a novel nanolitre osmometer provided data comparable to earlier studies which used other instruments. Osmolality in the dry eyed PMW group was higher than in the non dry eye group. In addition, a tendency towards less tear ferning in people aged over 40 years of age, regardless of their symptoms, was noted. Mild to moderate forms of dry eye show varying ferning grades. There was no significant correlation between tear osmolality and ferning patterns.

In Chapter 7 no difference was found in tear film lipocalin or lysozyme concentration between dry eyed and non dry eyed PMW, using two tear collection methods (capillary tear collection and an eyewash technique). Comparison of clinical signs, including non-invasive tear break up time and phenol red thread test, with lipocalin and lysozyme concentrations failed to reveal statistically significant correlations. The results showed significant inter subject tear variability. Trends of decreasing tear lipocalin and lysozyme levels in the symptomatic PMW group were detected, but statistical significance was not achieved using either tear collection method.

Chapter 8 showed that Conjunctival Impression Cytology (CIC) is a relatively simple, practical and minimally invasive technique to collect epithelial cells from the conjunctiva. The Millipore (MP) filter paper membrane used in the CIC procedure, when processed with either the TRIzol™ (TZ) RNA isolation technique or RNeasy™ Mini (RN) methods, are equally efficient for the isolation of high quality RNA from conjunctival cells collected *in situ*. The RN method was recommended, due to enhanced speed as well as on-column isolation and DNase digestion capabilities. For total protein

isolation, the use of CIC using MP membranes followed by immediate freezing and then extraction and processing with methods optimized by our laboratory, facilitates the collection of total protein from human conjunctival cells. Both methods will prove very useful to assess the expression of a variety of proteins involved in both normal and diseased conditions affecting the ocular surface. From a researchers' perspective, flash freezing of CIC membrane and then isolation of protein is recommended due to practical difficulties of isolating each sample immediately after collection.

Chapter 9 demonstrated the collection of tear samples and CIC samples from PM women to analyze the expression of MUC1 and MUC16. No difference was found in the expression of either MUC1 or MUC16 protein or mRNA expression between symptomatic PMW and controls. NITBUT values did not appear to be associated with MUC1 and MUC16 expression (protein or mRNA). Our results showed huge variability in MUC expression levels with each group, emphasizing the variability within subjects in the two groups studied. This may also be due, in part, to the grouping criteria (based on symptoms alone). Further research is required to investigate the potential use of biomarkers such as MUC1 and MUC16 in the characterization of dry eye disease especially in severe forms.

11 Future work

Technological advancements in instrumentation and analytical methods over the past 10 years have resulted in a better understanding of the pathogenesis of dry eye disease. However, even though there are many tests available to evaluate dry eye, there remains no "gold standard" measurement[1] or combination of tests available for dry eye diagnosis, with the contentious exception of tear film osmolality. Attempts are being made to devise minimally invasive techniques for tear film and ocular cell sampling to study dry eye.

The various studies undertaken within this PhD project did raise a number of interesting and worthwhile questions. A major concern that is raised when any dry eye study is conducted relates to the grouping criteria used to decide upon those who have dry eye, as this will clearly impact on the results obtained. Dry eye is generally considered to be a symptom-based disease. However, symptoms may be under-estimated[2] or over-estimated and is, obviously, very subjective.[2] It is therefore better to group participants based on both clinical signs and symptoms. However, even this approach may not be simple to undertake, as it is a well established fact that clinical signs and symptoms do not correlate well in dry eye, except for severe cases.

From a research standpoint, subject recruitment must include "strict" inclusion and exclusion criteria, with the incorporation of both clinical signs and symptoms, to avoid the "over lap" of non-dry eyed individuals with those with only mild disease. Studies with multiple visits to monitor dry eye over a period of time would be beneficial, as opposed to single visit studies which attempt to obtain many variables.

Various issues which were not addressed in this study would be valuable additions to the literature, on PMW who are not on HRT. These include:

- *Staining of the ocular surface with vital dyes*: This study design did not permit us to grade ocular surface staining, which is a valid criticism of this thesis. Future studies should investigate the staining obtained with both fluorescein and rose Bengal/lissamine green. These stains could then be graded using a variety of grading scales.[3-5]
- *Confocal microscopy to monitor corneal changes*: An increase in osmolality and inflammation are related to apoptosis and changes in the cellular structure of the surface of the eye, including the cornea and conjunctiva.[6, 7] The use of corneal confocal microscopy would be useful in understanding the cellular changes that occur in dry eye, and could be used to investigate any correlation between ocular surface staining and surface cell structural changes.
- *Evaluation of corneal cells:* Harvesting corneal epithelial cells using non-contact corneal irrigation chamber which enables non-invasive collection of epithelial cells from the corneal surface of human subjects[8-10] may be beneficial to study the structure of the sloughed off corneal epithelial cells under a microscope using different dyes in a group of dry eyed participants.
- *Conjunctival and corneal sensitivity assessment*: The issue of ocular surface sensitivity, especially corneal sensitivity, and its role in the development of dry eye symptoms requires further investigation, particularly in PMW. Symptomatic individuals have shown a decrease in dry eye symptoms with advancing age,[11] which

may be due to the decrease in corneal sensitivity.[12] On the contrary, increased corneal sensitivity in dry eye has also been noted.[13]

- *Analysis of meibomian gland secretions*: Androgen levels play an important role in dry eye disease, especially in Sjogren's syndrome. There is very limited information available about androgen levels and its impact on dry eye in PMW.[14-17] The majority of elderly women suffer from some form of meibomian gland dysfunction, which is a major causative factor in dry eye. Understanding the constituents of meibum and lipid within the tear film would prove to be very useful in the development of future dry eye therapies.
- *Analysis of cytokines*: With the evolution of the new definition of dry eye,[18] one area that is of current interest to dry eye researchers includes the inflammatory mediators involved in the pathogenesis of dry eye, including cytokines. Novel, user friendly, reliable and repeatable instruments that allow researchers to analyse cytokines, MMP's and other inflammatory mediators in tears and ocular surface cells will be beneficial to gain insight in this area of dry eye.
- *Analysis of mucins in severe dry eye*: In this thesis, focus was drawn towards soluble and membrane bound MUC 1 and MUC 16. Moreover, the participant pool in this thesis were divided symptomatically and the majority categorized themselves as having mild to moderate symptoms. It will be interesting to study other mucin species in more severe forms of dry eye. Studies of this nature may be beneficial in terms of potential secretagogues that can be used in the treatment of dry eye.

The DEWS report also gives a summary of specificity and sensitivity of dry eye tests and templates to perform dry eye tests. This can serve as a good starting point to

choose validated clinical and analytical tests in designing dry eye studies to explore different areas (both clinical and analytical) in this group of participants.

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Appendix A

OCULAR SURFACE DISEASE INDEX©

Please answer the following questions by checking the box that best represents your answer.
Have you experienced any of the following during <u>the last week</u>:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time
1. Eyes that are sensitive to light? ….					
2. Eyes that feel gritty? ……………					
3. Painful or sore eyes? ……………..					
4. Blurred vision? ……………………					
5. Poor vision?………………………..					

Have problems with your eyes limited you in performing any of the following during <u>the last week</u>:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
6. Reading? …………………….						
7. Driving at night? ..…………..						
8. Working with a computer or bank machine (ATM)? ………..						
9. Watching TV?..………………..						

Have your eyes felt uncomfortable in any of the following situations during <u>the last week</u>:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
10. Windy conditions? ………….						
11. Places or areas with low humidity (very dry)? ……..…						
12. Areas that are air conditioned?						

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Appendix B

SUBJECTIVE EVALUATION OF SYMPTOM OF DRYNESS

Please evaluate your ocular discomfort due to the symptom of “Dryness” on a scale of 0 (none) to 4 (severe). You may use the following descriptions to assist in your score.

None (0)	=	I do not have this symptom
Trace (1)	=	I seldom notice this symptom, and it does not make me uncomfortable.
Mild (2)	=	I sometimes notice this symptom, it does make me uncomfortable, but it does not interfere with my activities.
Moderate (3)	=	I frequently notice this symptom, it does make me uncomfortable, and it sometimes interferes with my activities.
Severe (4)	=	I always notice this symptom, it does make me uncomfortable, and it usually interferes with my activities.

Appendix C

DRY EYE QUESTIONNAIRE

Indiana DEQ

Patient Number: ________
Date ________
Time ________

DRY EYE QUESTIONNAIRE

1. Have you worn contact lenses at least 3 days a week, eight hours a day, in the past month?

   1 Yes (please stop and fill out the contact lens wearer questionnaire)
   2 No

2. If you have worn contact lenses in the past, have you ever worn any of the following types of lenses?

	Yes	No
a. Rigid gas permeable	1	2
b. Soft daily wear (lenses replaced after 1 year or longer)	1	2
c. Frequent replacement (lenses replaced after 1 month or longer)	1	2
d. Disposable (lenses replaced daily or every 2 weeks)	1	2
e. Extended wear (lenses worn overnight)	1	2

3. How important was each of the following issues in your decision to stop wearing contact lenses?

	Not at All Important				Very Important	Not Applicable
a. I never got used to the lenses	1	2	3	4	5	0
b. The lenses were uncomfortable all day	1	2	3	4	5	0
c. The lenses were most uncomfortable when first put in	1	2	3	4	5	0
d. The lenses became more uncomfortable later in the day	1	2	3	4	5	0
e. My eyes felt dry	1	2	3	4	5	0
f. The lenses felt scratchy and irritating	1	2	3	4	5	0
g. My vision was not clear enough	1	2	3	4	5	0
h. My vision fluctuated throughout the day	1	2	3	4	5	0
i. Wearing contact lenses was too much trouble	1	2	3	4	5	0
j. Contact lenses were too expensive	1	2	3	4	5	0
k. I couldn't put the lenses in easily	1	2	3	4	5	0
l. I couldn't sleep in the lenses	1	2	3	4	5	0
m. I had red or infected eyes	1	2	3	4	5	0
n. Other reason (please specify below)	1	2	3	4	5	0

____

3d. What is your age?

____

3e. What is your gender?

1 Male **2** Female

4. Questions about **EYE COMFORT**:

a. During a typical day in the past week, how often did your eyes feel uncomfortable?

- **1** Never (please skip to question 5)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When your eyes felt discomfort, how intense was this feeling of discomfort…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

5. Questions about **EYE DRYNESS**:

a. During a typical day in the past week, how often did your eyes feel dry?

- **1** Never (please skip to question 6)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When your eyes felt dry, how intense was this feeling of dryness…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

6. Questions about **BLURRY VISION**:

a. During a typical day in the past week, how often did your vision change between clear and blurry? (This is often described as "foggy or steamy vision that clears up when you blink.")

- **1** Never (please skip to question 7)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When your vision was blurry, how noticeable was this blurry vision…

b. Within the first two hours of getting up in the morning?

Not at All Noticeable				Very Noticeable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

(2)

c. In the middle of the day?

Not at All Noticeable				Very Noticeable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Noticeable				Very Noticeable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

7. Questions about **EYE SORENESS** and **IRRITATION**:

a. During a typical day in the past week, how often did your eyes feel sore and irritated?

1 Never (please skip to question 8)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt sore and irritated, how intense was this feeling of soreness…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

8. Questions about **EYE GRITTINESS** and **SCRATCHINESS**:

a. During a typical day in the past week, how often did your eyes feel gritty and scratchy (as if a piece of sand was in your eye)?

1 Never (please skip to question 9)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt gritty and scratchy, how intense was this feeling of grittiness and scratchiness…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

9. Questions about **SOMETHING IN YOUR EYE**:

a. During a typical day in the past week, how often did you have the feeling that "something" was in your eye (as if a piece of sand was in your eye)?

- **1** Never (please skip to question 10)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When you felt as if something was in your eye, how intense was this feeling…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

10. Questions about **EYE BURNING** and **STINGING**:

a. During a typical day in the past week, how often were your eyes burning and stinging?

- **1** Never (please skip to question 10)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When your eyes were burning and stinging, how intense was this feeling of burning and stinging…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

11. Questions about **LIGHT SENSITIVITY**:

a. During a typical day in the past week, how often did your eyes feel unusually sensitive to bright lights?

1 Never (please skip to question 12a)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt unusually sensitive to bright lights, how intense was this unusual sensitivity…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

12. Questions about **EYE ITCHING**:

a. During a typical day in the past week, how often did your eyes itch?

1 Never (please skip to question 13a)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt itchy, how intense was this feeling of itchiness…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

13a. How often, in a typical day in the past week, did your eyes *bother you so much* that you felt as if you needed to stop whatever you were doing and close your eyes? Please choose the answer that is closest to your situation.

1 Never (please skip to question 13)
2 Infrequently
3 Frequently
4 Constantly
5 Daily
6 Several times a day

13b. When your eyes bothered you so much that you felt as if you needed to *stop* what you were doing and *close your eyes*, how much did each of the following symptoms contribute to this feeling?

	Not At All				Very Much	Not Sure
a. Eye discomfort	**1**	**2**	**3**	**4**	**5**	**0**
b. Eye dryness	**1**	**2**	**3**	**4**	**5**	**0**
c. Eye soreness	**1**	**2**	**3**	**4**	**5**	**0**
d. Eye grittiness and scratchiness	**1**	**2**	**3**	**4**	**5**	**0**
e. Eye burning and stinging	**1**	**2**	**3**	**4**	**5**	**0**
f. Light sensitivity	**1**	**2**	**3**	**4**	**5**	**0**
g. Eye itching	**1**	**2**	**3**	**4**	**5**	**0**

13c. How often during past month did your eyes bother you so much that you **did** stop what you were doing and close your eyes? Please choose the answer that is closest to your situation.

1 Never
2 Less than once a week
3 Weekly
4 Several times a week
5 Daily
6 Several times a day

14a. How many hours during a typical workday do you use a computer?

__________ hours

14b. How many hours during a typical day when you're not working do you use a computer?

__________ hours

15. Are you currently taking any of the following medications?

	Yes	No
a. Thyroid medications	1	2
b. Blood pressure medications	1	2
c. Diabetes medications	1	2
d. Diuretics	1	2
e. Accutane	1	2
f. Heart condition medications	1	2
g. Depression medications	1	2
h. Ulcer medications	1	2
i. Oral contraceptives	1	2

16a. Do you have any of the following allergies?

	Yes	No	Don't Know
a. Seasonal allergies (Hayfever, "sinus")	1	2	3
b. Skin allergies (Dermatitis)	1	2	3
c. Asthma	1	2	3
d. Allergies to animals	1	2	3
e. Allergies to pollen & mold	1	2	3
f. Allergies to food	1	2	3
g. Allergies affecting your eyes	1	2	3
h. Allergies to contact lens solutions	1	2	3
i. Allergies to eye drops	1	2	3

16b. If you take any of the following medications for your allergies, how often do you take them?

	Less than once a week	Once a week	2-3 times a week	Daily	More than once daily	Do Not Take
a. Antihistamine pills or liquid	1	2	3	4	5	0
b. Antihistamine eye drops	1	2	3	4	5	0
c. Decongestant pills or liquid	1	2	3	4	5	0
d. Decongestant eye drops	1	2	3	4	5	0
e. nasal inhalants	1	2	3	4	5	0

17. In the past month, how often have you experienced dryness of the mouth, nose or vagina? Please choose the answer that is closest to your situation

1 Never
2 Less than once a week
3 Weekly
4 Several times a week
5 Daily

18a. Have you been told you have dry eye(s)?

1 Yes **2** No **3** Don't Know

18b. If you use any of the following treatments, how much help do they provide?

	No Help <u>At All</u>				Complete <u>"Cure"</u>	Do Not <u>Use</u>
a. Artificial tears	**1**	**2**	**3**	**4**	**5**	**0**
b. Contact lens rewetting drops	**1**	**2**	**3**	**4**	**5**	**0**
c. Warm compresses and/or eyelid cleaning	**1**	**2**	**3**	**4**	**5**	**0**
d. Punctal plugs or cauterization.............	**1**	**2**	**3**	**4**	**5**	**0**
e. Other (please specify below)	**1**	**2**	**3**	**4**	**5**	**0**

____

19. Do you think you have dry eye(s)?

1 Yes **2** No **3** Don't Know

20. Have you used artificial tears in the last month?

1 Yes (please answer questions 21, 22, and 23 below)
2 No (***Thank You,*** that completes this survey)
3 Don't Know (***Thank You,*** that completes this survey)

21. In the last month, about how many times per day did you use artificial tears or contact lenses?

__________ times

22. In the last month, about how many bottles of artificial tears did you use?

__________ bottles

23. In the last month, about how much money did you spend on the purchase of artificial tears?

__________ dollars

Appendix D

Total protein isolation from the Conjunctival Impression Cytology (CIC) samples

Total protein isolation from the CIC samples was performed for sample IDs # 1 to 86.

1. Four tubes (each tube contained 2 membranes taken from the impression cytology of the left eye) were removed from -80ºC freezer and placed on wet ice.
2. One membrane from the tube was removed and placed with cell side facing up on a glass slide. 5µL of extraction buffer (50 mM Tris, 2% SDS, 1X Protease Inhibitor™, (PI) (Roche Diagnostics)) was added to the membrane.
3. The second membrane was placed on top of first membrane and an additional 5µL of extraction buffer was added onto top membrane.
4. Using a #10 carbon steel scalpel blade, the membranes were cut into small pieces, (1-2 mm square) and the cut membrane pieces were placed in a 600µL eppendorf tube (Axygen MAXYMum Recovery; Axygen Scientific, Inc, Union City, CA).
5. The eppendorf tube was placed on wet ice until the remaining samples were processed (all the four samples were coded and placed in separate tubes). An additional 50 µL of extraction buffer was added to all tubes.
6. Following a 10 second vortexing, the tubes were placed into boiling water for 10 minutes. The tubes were vortexed briefly for an additional five seconds and the tubes were then placed into cap removed, 1.7 mL eppendorf tubes (Axygen MAXYMum Recovery; Axygen Scientific, Inc, Union City, CA).
7. The caps were pierced to release pressure. While the smaller tube was inside the larger tube, the bottom of smaller tube was pierced twice with an 18g needle.

8. The samples were spun at 12,000 relative centrifugal force (rcf) for 6 minutes.
9. An additional 20 μL of extraction buffer was added onto IC membrane of each tube and the samples were re-spun for 6 minutes at 12,000 rcf (VWR Mini Vortexer, VWR International, USA).
10. The smaller tubes were removed; the supernatant was collected in the large eppendorf tube. Approximately 5μL of the supernatant was used for protein assay and the remaining sample was aliquoted into a separate tube and stored at $-80^{o}C$ for Western blot analysis.

Appendix E

Total protein determination for tear and CIC samples

Total protein determination was performed using the BioRad DC™ protein assay (Cat # 500-0116). The various steps involved in the procedure are described below:

Microplate DC Assay Protocol:

1. Sample preparation (each sample contained the following):
   a. Tears: 0.5µL of tears was added to 9.5µL of Milli-Q water.
   b. Eye Wash: 5µL of eye wash was added to 5µL of Milli-Q water.
   c. IC supernatant: 5µL of IC supernatant was added to 5µL of Milli-Q water.
2. Preparation of working reagents as per manufacturer's guidelines:
   a. For those samples that contained SDS, 20µL of Reagent S was added to each mL of Reagent A that was required (= Reagent A').
   b. If the samples did not contain the detergent (SDS), step #2a was omitted and Reagent A as supplied was used.
3. Prediluted dilutions of Pierce BSA Protein Assay Standard Kit (Pierce Cat# 23208) were used. There are 7 dilutions, ranging from 125 µg/mL to 2000 µg/mL, with Milli-Q water being used for the zero. A standard curve was platted each time the assay was performed.
4. Triplicate 5µL standards or duplicate 5µL diluted samples were pipetted into a clean, dry 96 well microplate.
5. 25µL of Reagent A' or Reagent A (see note from step 2) was added into each well.

6. 200µL Reagent B was added into each well. Using the microplate reader (96 well Micro-Well plates by NUNC, VWR International) mixing function, the plates were gently mixed for 5 seconds.
7. Following 15 minutes, absorbances were read at 750 nm on a Multiskan Microplate Spectrphotometer (Thermo Labsystems Cat# 28010).
8. Graphs were plotted on an excel sheet from the standard readings.
9. Using the standard linear regression equation, the amount of protein per well and then per µL of sample was calculated.

Appendix F

Quantification of individual lacrimal gland tear proteins (lysozyme and lactoferrin)

Electrophoresis and immunoblotting for Lysozyme:

1. Human neutrophil lysozyme (Sigma #L-8402; Lot#104K1040) standards of 10, 7, 4, and 1 ng/µL lysozyme were prepared in Tear Dilution Buffer (TDB; 10mM Tris (pH 8.0), 1 mM EDTA, 0.9% NaCl) and Gel Loading Buffer (GLB; 60mM Tris (pH 6.8), 2% glycerol, 2% SDS, and 0.01% bromophenol blue).
2. Samples were diluted with an equal volume of Laemmli's buffer (50mM Tris pH6.8, 2.5% glycerol, 2.5% SDS, and 0.03% bromophenol blue) and frozen. These were called stock tears.
3. Samples were briefly warmed to room temperature, mixed, and 0.5µL was removed from each and diluted with GLB to prepare sample stocks of 25 ng/µL for eye wash and 50 ng/µL for tears.
4. The stocks were further diluted to 25, 15, or 7.5 ng/µL as appropriate for electrophoresis.
5. Prepared sample extracts and standards were boiled for five minutes after which 0.8µl each was loaded onto a parafilm-covered template for loading of 12 X 0.3µl combs by capillary action.
6. All samples were subjected to SDS-PAGE on precast 10-15% gradient gels with a 13 mm stacking zone and 32 mm gradient zone on an automated minigel system (Amersham Pharmacia Biotech PhastSystem™), using the manufacturer's specified conditions.

7. Standard curves were run on each Western blot so that four points falling within the linear range of detection were produced, to facilitate regression analysis of sample extracts.
8. Once separated, proteins were analyzed via Western blotting. The gels were transferred onto PVDF membranes (BioRad, 0.2μm) pre-equilibrated with transfer buffer (20mM Tris, 150mM glycine; 10% (v/v) methanol) using a PhastSystem™ following the manufacturer's specified conditions.
9. Once transfer was complete, the PVDF membranes were dried for 10 minutes at room temperature and then baked for 60 minutes at 50ºC, allowed to cool for 5-10 minutes, briefly rewetted with methanol, and then blocked overnight (4ºC, with shaking) with 20% (w/v) skim milk powder in Tris-buffered saline (TBS-t; 50mM Tris; 100mM NaCl pH 7.4, 0.05% (v/v) Tween®-20).
10. Following blocking, membranes were washed 3 times, each 5 minutes in duration in 50 mL of TBS-t. The membranes were probed with primary antibody at room temperature with shaking for 2 hours [1:1000 polyclonal rabbit anti-human lysozyme (Cedarlane #RAHU/LYS/7S) in 5% blocking solution].
11. Blots were washed with TBS-t 3 x 5 minutes (50mL each) and then incubated with secondary antibody for 1 hour [1:20 000 goat anti-rabbit IgG-HRP (Sigma A-0545), room temperature with shaking].
12. Blots were washed 5 x 5 minutes (50mL TBS-T each), and 1 x 1 minute with TBS (no Tween).
13. Bound antibody was visualized by enhanced chemiluminescence (ECL Plus®) detection and results were captured with a Storm840® Imaging System

(Molecular Dynamics, Sunnyvale, CA, USA). Data was used to generate a regression plot upon which lysozyme concentration in samples was extrapolated.

Electrophoresis and immunoblotting for Lipocalin:

1. Standards for Lipocalin-1 were pooled tear samples from non dry eyed volunteers (evaluated by questionnaire).
2. Pooled tears were initially diluted with reduced 2X Laemmli's in a 1:1 ratio and then diluted with 1X reduced Laemmli's buffer to 30ng/μL, 20ng/μL, 10ng/μL, and 5 ng/μL.
3. Tear samples were diluted with an equal volume of Laemmli's buffer (50mM Tris pH6.8, 2.5% glycerol, 2.5% SDS, and 0.03% bromophenol blue) and frozen. This was called stock tears and stock eyewash.
4. Tear samples were diluted to 10ng/μL and eye wash samples were diluted to 15ng/μL. (NOTE: If a Lipocalin concentration from a sample did not lie within the pooled tears standard range, then a second blot was done with more or less total protein depending upon the previous result.)
5. All samples were subjected to SDS-PAGE on precast 10-15% gradient gels with a 13 mm stacking zone and 32 mm gradient zone on an automated minigel system (Amersham Pharmacia Biotech PhastSystem™) using the manufacturer's specified conditions.
6. Standard curves were run on each Western blot so that four points falling within the linear range of detection were produced, to facilitate regression analysis of sample extracts.

7. Once separated, proteins were analyzed via Western blotting. The gels were transferred onto PVDF membranes (BioRad, 0.2μm) equilibrated with transfer buffer (20mM Tris, 150mM glycine; 10% (v/v) methanol) using a PhastSystem™ following the manufacturer's specified conditions.
8. After blocking overnight with 10% blotto(5g skim milk, 50mL TBS –T), 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.
9. This was followed by a 1 hour incubation with peroxidase-conjugated goat anti-mouse secondary antibody (1:10 000) diluted in TBS-T.
10. Blots were washed 5 x 5min (50mL TBS-T each), and 1 x 1min with TBS (no Tween).
11. Bound antibody was visualized by enhanced chemiluminescence (ECL Plus®) detection and results were captured with a Storm840® Imaging System (Molecular Dynamics, Sunnyvale, CA, USA). Data was used to generate a regression plot upon which lipocalin concentration in samples was extrapolated.

Appendix G

Isolation of RNA from Conjunctival Impression Cytology (CIC) samples

RNA was isolated from right eye CIC samples using a commercially available RNeasy Mini Kit™ (Qiagen Cat # 74106) and the DNAse step was performed using the RNase-Free™ DNase Set (Qiagen Cat# 79254). The step-wise procedure is described below:

RNA isolation using commercially available RNeasy Mini Kit:

RNA was isolated from the samples according to manufacturer's guidelines with several modifications as explained below:

1. Samples collected via impression cytology (as described in 3.11.2 of methods chapter) were removed from -80°C storage and allowed to thaw. These samples contained the Millipore membranes soaking in 1 mL of RLT buffer (+10 µL of β-mercaptoethanol). All thawed samples were vortexed well for 30 seconds. Up to four samples were processed at the same time.
2. The Millipore membranes were removed from the RLT buffer using sterile forceps and the samples were vortexed again for 15 seconds.
3. 20 gauge needles were used to homogenise each of the samples by passing the samples several times (at least 20 times) through the syringe.
4. One volume (1 mL) of 70% ethanol was added to the homogenized samples and vortexed again for 15 seconds.

5. 700µL of the sample was added to the RNeasy mini spin column and centrifuged (VWR Mini Vortexer, VWR International, USA) for 15 seconds at 8000g.
6. Flow-through was discarded and step 5 was repeated with the remaining volume of the samples.

DNAse step

1. 350µL of buffer RW1 was pipetted to the above sample and centrifuged for 15 seconds at 8000g. Flow-through was discarded.
2. 22µL of DNAse I stock solution was added to 154µL of Buffer RDD and mixed gently by careful inversion.
3. 80µL of DNAse I was directly added on to the spin column membrane.
4. The spin column was incubated with DNAse I for 15 minutes at room temperature.
5. 350µL of buffer RW1 was added to the spin column and centrifuged for 15 seconds at 8000g. Flow-through was discarded.
6. 500µL of buffer RPE was then added to the column and centrifuged for 15 seconds at 8000g and the flow-through was discarded.
7. A second RPE buffer wash was performed by adding an additional 500µL of the buffer to the column and this was centrifuged for 2 minutes at 8000g to dry the membrane.
8. The dry RNeasy mini spin column was placed in a new 2mL collection tube and the old collection tube was discarded with the flow-through (care was taken not to allow the column to touch flow-through).

9. The new 2mL collection tube with the dry column was then centrifuged for additional 15 seconds at 8000g to completely dry the membrane.
10. The column was transferred again in to a new 1.5mL collection tube. The old 2mL collection tube was discarded.
11. 40µL of RNAse free water was pipetted into the column and centrifuged for 1 minute at 8000g to collect RNA samples.
12. The sample flow through was carefully pippetted out into a new 1.5mL collection tube and the RNA sample was immediately placed in a -80°C freezer for storage until use for RT-PCR.

Appendix H

Staining Conjunctival Impression Cytology (CIC) Samples

1. Samples (CIC membranes) were fixed in 95% ethanol and were hydrated for 1-2 minutes in distilled water (ddH2O)
2. Samples were then soaked in Gill's hematoxylin for 2 minutes.
3. Samples were then repeatedly rinsed in ddH2O for several dips for at least 3 minutes.
4. Samples were differentiated in 0.5% hydrochloric acid in 70% ethanol (0.5 mL/100mL) until nuclei were distinct against a pale blue cytoplasm.
5. Samples were washed gently in water for 1 -2 minutes until nuclei were clear blue.
6. Samples were dipped in ammonium hydroxide water (bluing agent consisted of 1.5mL NH4OH in 750 mL dH2O) several times and then rinsed in ddH2O for 1 minute.
7. The membrane was stained in eosin for 1-2 mins (Eosin stain recipe: eosin Y 1.0 g + 1000 mL 70% EtoH + 5 mL glacial acetic acid). Volume to be used when staining were diluted with equal volume of 70% ethanol and 2-3 drops of glacial acetic acid was added (For example: To obtain10 mL for stain, 5 mL of the eosin and 5 mL of 70% ethanol and 2 to 3 of drops of glacial acetic acid were used).
8. Sample was rinsed in 95% ethanol for a 3 to 4 dips. The samples were then dehydrated through 1 X 95% and 2 X 100% ethanol (1 min each with agitation).
9. The membrane was placed in xylene and placed on a slide and mounted.
10. The cells were evaluated under a microscope set at 40X magnification (Zeiss Axiovert 40 CFL, Carl Zeiss, Göttingen, Germany).

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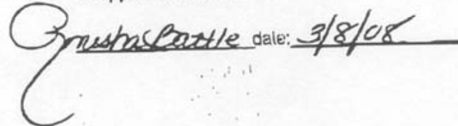

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